Evolution of the Arthropod Mandible:

a molecular developmental perspective

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Declaration

I, Joshua Frederick Coulcher, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The mandible is thought to have evolved once in the ancestor to the mandibulate arthropods; the insects, crustaceans and myriapods. If the mandible is a homologous structure, it suggests that there will be shared developmental genes required to pattern the mandible in different species. As a representative of mandibulate arthropods, the red flour beetle *Tribolium castanem* was chosen to study genes required to pattern the mandible. This study show that the *Tribolium* orthologue of *cap'n'collar* (*Tc cnc*) patterns the mandible of *Tribolium*. Loss of *Tc cnc* function by RNA interference (RNAi) results in a transformation of the mandible to maxillary identity and deletion of the labrum. Analysis of gene expression by *in situ* hybridisation shows that *Tc cnc* represses the *Tribolium* orthologues of the Hox genes *proboscipedia (pb)* and *Deformed (Dfd),* which pattern the maxillary appendage.

Similar expression patterns of *cnc, Dfd* and *pb* homologues in mandibulate arthropods suggests that the functions of these genes are conserved. As the mandible has evolved from a maxilla-like precursor in the ancestor to all mandibulate arthropods, the manner in which *Tc cnc* differentiates the mandible from a maxilla in *Tribolium* recapitulates the evolution of the mandible from a maxilla-like precursor.

An orthologue of *cnc* was cloned from the spider *Achaearanaea tepidariorum,* chosen as an outgroup to the mandibulate arthropods, but no evidence of a developmental role was discovered.

Study of the expression of genetic markers for appendage segments shows that the biting edge of the mandible is derived from one endite, and the mandible is divided into a subcoxa and coxa which are also present in the maxillary, labial and leg appendages. There are significant similarities in the expression of genetic markers that presumably indicate serial homology of the subcoxa and coxa of the mandible to the subcoxa and coxa of other appendages.

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Chapter 1: Introduction

1.1 General introduction

The arthropod mandible is an appendage adapted for biting and chewing and is present in three arthropod groups, the insects, crustaceans and myriapods (millipedes and centipedes). The mandibulate arthropods constitute the majority of animals both in terms of species diversity and biomass on this planet. The evolution of the mandible is therefore of particular interest as it is an evolutionary novelty of such an important and diverse group of animals.

There are many different types of mandible, but the characteristic that is shared by most mandibles is the presence of a functional biting edge made up of an incisor and a molar process (see fig.1.1). A study of the fossil record shows that the mandible, along with most appendages, has evolved from a particular type of limb, which is called the biramous limb. The biting edge of the mandible has evolved from the base of the leg (more specifically from a structure called an endite). This fact is most obvious in some types of crustacean mandibles which have a leg-like palp attached to the segment that carries the biting edge (see fig.1.1F).

If the mandible evolved once in the ancestor to the insects, crustaceans and myriapods, it might be expected that there would be some similar genes involved in development that would be shared by these different groups. Understanding what these genes are, how they function and whether they are shared by diverse mandibulates could tell us about mandible evolution: it could tell us the mandible of insects, crustaceans and myriapods had a common origin and it could also tell us about how the mandible has evolved from a leg-like appendage.

The overall aim of my research thesis is to understand the evolution of the mandible by studying its embryonic development from a molecular perspective. An understanding of the development of the mandible is best achieved initially through functional study of patterning genes in a model organism. From this position it is easier to study the role of genes in more diverse taxa that are more difficult to study.

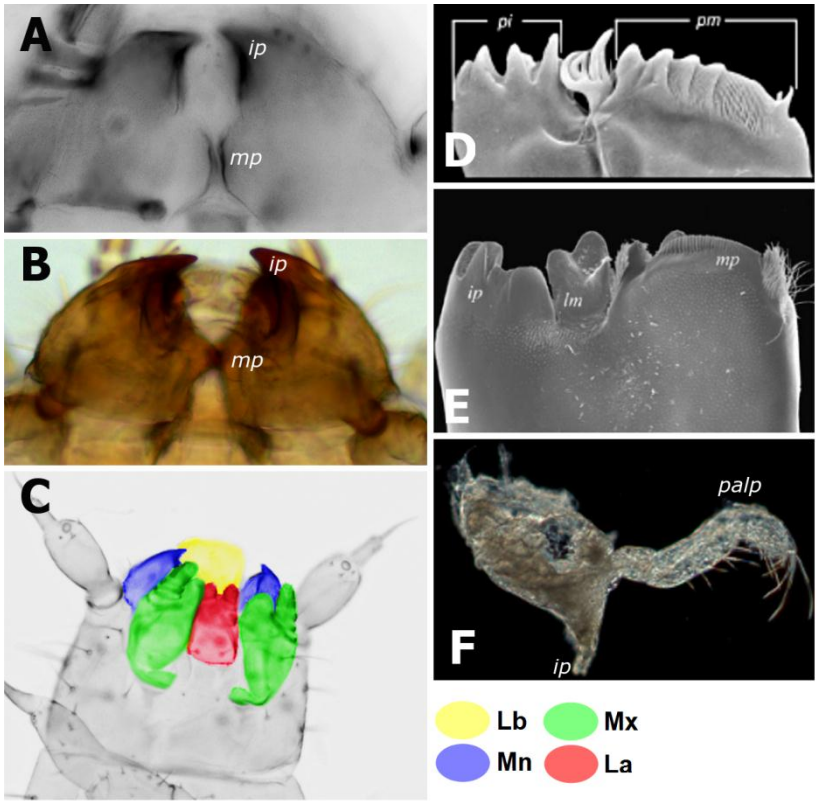

Fig.1.1. The arthropod mandible. The gnathal edge is comprised of an incisor (ip) and molar (mp) process forming the gnathal edge. A movable plate-like structure, the lacinia mobilis (lm) is present on some mandibles. (A-B) *Tribolium* larva are in possession of a characteristic mandibular gnathal edge (A) Close up of a first instar *Tribolium* mandible visualized by fluorescence microscopy (B) Close up of a cuticle preparation of a later stage instar *Tribolium* larval mandible. (C) Cuticle preparation of *Tribolium* larval head with highlighted mouthpart and labral structures: Labrum (yellow), mandible (blue), maxilla (green), labial appendages (red). (D) SEM of the gnathal edge of a symphylan myriapod *Hanseniella (Edgecombe et al., 2003)* (E) SEM of the gnathal edge of a peracarid crustacean *Gnathophausia gracilis* (Richter, 2004). (F) Mandible of *Gammarus,* an amphipod crustacean, with a telopodite derived palp (palp)(Browne and Patel, 2000).

The majority of research into the function of genes patterning arthropod gnathal appendages has focused on insects that possess highly derived, mouthparts in which the mandible has been converted to a proboscis in the fruitfly *Drosophila melanogaster* and a stylet in the milkweed bug *Oncopeltus fasciatus.* There has been no functional study of genes necessary to pattern the mandible in a mandibulate arthropod. To achieve this I therefore chose a model organism, the red flour beetle *Tribolium castaneum*, which possesses a typical mandible with primitive characteristics. By understanding important mandible patterning genes in this species and comparing them to other taxa across Arthropoda, it is hoped that similarities to other mandibulate arthropods can be discovered to show that the mandible evolved once in the ancestor to the mandibulate arthropods. Also, it is hoped that the manner in which the developmental genes function in *Tribolium* will provide some clues about

how the mandible has evolved, say for example from the starting point of another type of appendage and whether there are structures still present on the mandible which are similar to other parts of other appendages.

To complement embryological, morphological and palaeontological studies, I wanted to investigate how the developing *Tribolium* mandible is constructed in the embryo by studying the expression of genetic markers. By dividing the developing mandibular lobe into sub-structures (like the molar and incisor processes) and comparing them to similar structures on other appendages, any similarities observed could be suggestive of serial homology. Another important goal was to identify the genes which are necessary to pattern the mandible of *Tribolium.* By comparing the expression of these genes to their homologs in other mandibulate arthropods and non-mandibulate arthropods, the manner in which the mandible is patterned in *Tribolium* could demonstrate that the mandible is a homologous structure of mandibulate arthropods and could provide clues as to how the mandible has evolved from another type of appendage, for example from a leg or another type of mouthpart through changes to the genetic pathways that pattern them.

The first half of this introduction will present conclusions from subjects outside of molecular development. The purpose of this is to present ideas that form the basis of many assumptions that have informed the hypotheses and the design of the experiments revealed in later chapters.

Study of fossils of the likely ancestors to the arthropod lineage has shown that all post-antennal arthropod appendages have evolved from a particular type of limb known as a biramous limb. This is a particular limb structure present in the earliest representatives of true arthropods. The evidence for the origin of the biramous limb will be discussed.

As with all arthropod appendages, mandibles vary greatly according to the natural history of the particular species it is attached to; I provide an overview of the diversity of mandibular structures, such as the presence of palps, segmentation, attachment points (condyles) and the gnathal edge will be presented. Despite this diversity, the defining character of the mandible that distinguishes it from other appendages is the gnathal edge which is thought to be a homologous structure in insects, crustaceans and myriapods. The gnathal edge is thought to be derived from the endite of a biramous limb.

The insects, crustaceans and myriapods are hypothesized to form a monophyletic group called Mandibulata. Support for a monophyletic grouping of the arthropods and for grouping the insects with the crustaceans is strong. The position of the myriapods is more problematic and has implications regarding the evolution of the mandible. The phylogenetic evidence both for and against acceptance of Mandibulata will be summarized.

A hypothetical outline of mandible and maxilla evolution from an ancestral two-segmented protopodite will be presented. In this evolutionary scenario, the primitive mandibular gnathal edge is hypothesized to reside on the proximal-most segment of the mandible, the coxa. An alternative hypothesis will also be presented that suggests the primitive mandible possesses a subcoxal segment.

In order to study the developing embryonic mandible, it is necessary to use genetic markers. The second half of this introduction will present relevant background information from molecular development of embryos from diverse arthropod taxa. One conserved set of genes, the proximal-distal (PD) domain genes, are required to pattern the PD axis of all arthropod appendages and are useful to study the developing PD axis. The Notch signalling pathway is involved in patterning arthropod appendage segment boundaries.

Another class of genes, the Hox genes, are required to pattern segments along the anterior-posterior axis of the body. The conserved expression domains of Hox genes have shown that the homologous segment to the mandibular segment is the first leg segment of chelicerates.

Whilst *Drosophila* genetics is a useful resource for mining of candidate developmental genes, it is not suitable to study as a model organism for mandible development as it is lacking mandibular appendages. One gene, however - *cap'n'collar* - is required to pattern mandibular segment derived structures in *Drosophila* embryos. The evidence for this will be summarized.

Tribolium is a useful model organism to study embryonic mandible development as, unlike fruit flies, it possesses the primitive character of the mandible that distinguishes the mandible from other appendages, the gnathal edge. A summary of what is known about the mandible patterning genes in *Tribolium* will be presented.

Having presented a summary of the themes introduced above, specific questions that will be addressed experimentally in each chapter will be outlined.

1.2 Evolution of the biramous limb

In order to account for diversity of mandibles and other arthropod appendages and make sense of their evolutionary history, it is necessary to study the fossil record. The study of fossils is important for understanding ancestral character states and how these characters have evolved as it provides examples of appendages that actually existed in ancestral arthropods. Study of Cambrian fossils has shown that there were two types of limbs present in the ancestor to all arthropods which have subsequently evolved into all other types. They are the articulated antenna and, more posteriorly, the biramous limb. These serially homologous biramous limbs were present on each segment posterior to the antenna in numerous arthropods during the Cambrian. It is therefore clear that every post-antennal appendage has evolved from a biramous limb (Boxshall, 2004; Waloszek et al., 2007; Chen, 2009).

The articulated antenna is the most anterior appendage, homologous to the jaw of onychophorans, and is present in trilobites, myriapods and pancrustaceans. In the chelicerate lineage the antennae have probably been modified to form the chelicerae. By contrast the biramous limb is a complex appendage comprised of three parts: the protopodite, exopodite and the telopodite. The term biramous means that the limb has two axis or branches: the telopodite and exopodite. These two branches, or rami, are attached to a proximal structure called the protopodite. Structures called endites are often present on the medial/ventral side of the protopodite (see fig. 1.2D). The protopodite may also possess unsegmented outgrowths/branches called exites on the dorsal part of the protopodite.

Endites are convex structures that possess spines or bristles that have either a sensory function or are involved in the manipulation and processing of food. If the endite covers the entire medial side of the segment then it is called a gnathobase. The gnathobase is derived from the most proximal endite situated adjacent to a food groove leading to the mouth with a role in feeding. The mandible gnathal edge is derived from an endite. It is not hard to imagine derivation of the mandible from an existing structure on the proximal part of the limb that is already involved in feeding (Boxshall, 2004).

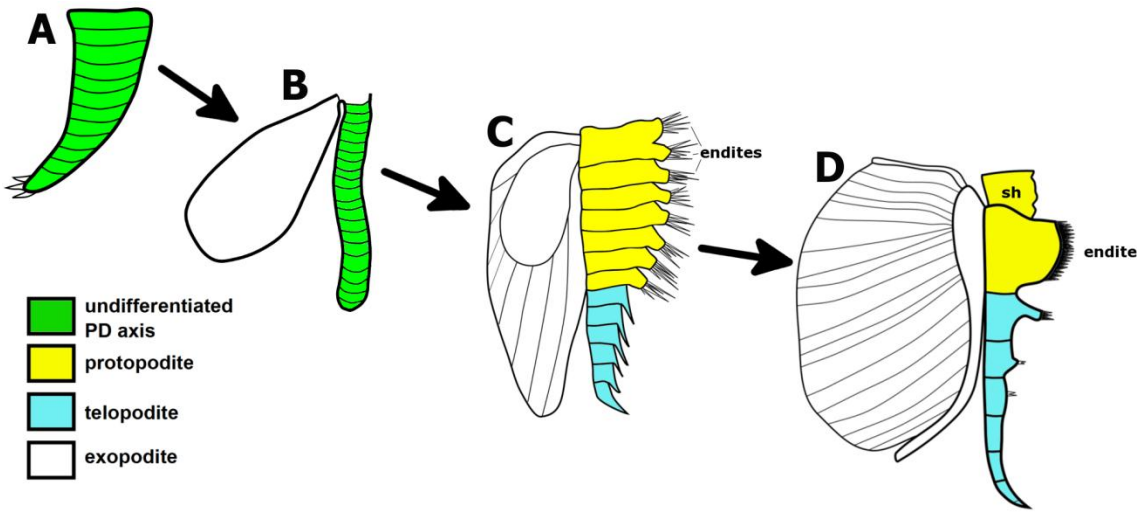

Fig.1.2. Evolution of the biramous limb in the stem lineage of Euarthropoda from a monopodial limb. Undifferentiated PD axes are highlighted in green. The telopodite is highlighted blue, the protopodite is highlighted in yellow, the exopodite is white. (A) Monopodial limb with annulations represented by the lobopodian *Aysheaia.* (B) Simple biramous limb divided into exopodite and an undifferentiated PD axis in proarthropods such as *Shankouia zhenghei, Fuxianghuia protensa, Chengjiangcaris longiformis.* (C) Multisegmented limb with the PD axis differentiated into a segmented protopodite and telopodite as represented by *Canadapsis laevigata.* Endites are present on the medial surface. Other examples of undifferentiated and multisegmented biramous limbs were present in the megacheirans (possible stem lineage Chelicerates) *Leachoilia, Fortiforceps,* and *Tanglangia*. More differentiated multisegmented limbs were present in fossils like *Ercaia miuscule, Cindarella eucalla,* and *Saperion glumaceum*. Biramous limbs with differentiation of the P/D axis into clear protopodite and telopodite divisions are present in the trilobite like Naroia and Euarthropods (Chen, 2009). (D) Differentiated limb with an unsegmented protopodite with a single well-developed endite as represented by the trilobite-like *Naraoia* and possibly the ancestral state of all arthropod post-antennal appendages. The unsegmented protopodite is attached to a shaft (sh).

The mandible is a post-antennulary appendage and it follows that it has evolved from a biramous limb. The ancestral mandible was likely to be a biramous limb, with two palps, and a gnathal edge (a modified endite) present on the base of the appendage. This gnathal edge would have probably had an incisor and molar processes.

Evolution of simple biramous limbs

The gradual evolution of biramous limbs from a simple unsegmented lobe-like monopodial limb is evident in the Cambrian fossil record and examples of each stage of limb development are present in different Cambrian arthropods, which is shown in fig.1.2 (Boxshall, 2004; Waloszek et al., 2007; Chen, 2009). The evolution of the arthropod limb progresses from simple lobes or annulated rod-like limbs present in lobopodians or tardipolypods through stem-lineage groups to crown group Euarthropoda, the true arthropods, in possession of differentiated biramous limbs (Waloszek et al., 2007; Chen, 2009; Edgecombe, 2010).

The lobopodians are stem lineage panarthropods, and may well be the ancestors to all arthropods and onychophorans. The lobopodians possess monopodial limbs (see fig.1.2A) that are homologous to all appendages of extant arthropods[1] (Waloszek et al., 2007; Budd and Telford, 2009; Chen, 2009). Members of one extant clade, the Onychophora, resemble Cambrian lobopodians and possess lobopodial limbs, however, onychophorans also possess numerous derived characters, such as terrestialisation and appendages such as jaws (not mandibles) and slime papillae (Budd and Telford 2009).

An intermediate stage of arthropod limb evolution from monopodial limbs to the biramous limb is present in some stem lineage arthropods (Waloszek et al., 2007; Chen, 2009; Edgecombe, 2010). Some of these stem lineage arthropods gave rise to the euarthropods which include the mandibulate arthropods, chelicerates and extinct taxa such as a trilobites, naraoiids and megacheirans. The limbs on numerous stem lineage arthropods are simple biramous limbs that consist of a simple segmented rod and a flap which are homologous to the telopodite and exopodite respectively. These limbs are repeated on each segment and are undifferentiated (see fig. 1.2B).

The final stage of biramous limb evolution involves the differentiation of the proximal and distal axis of the limb and the development of endites (see fig. 1.2C,D). The differentiation of the proximal part of the arthropod biramous limb with the addition of endites was probably associated with a change in diet and was a key event in the evolution of the Euarthropoda (Chen, 2009).

Endites on the protopodite

The protopodite is the proximal part of the biramous limb that is attached to the body. The protopodite is defined as the proximal part of the limb to which the telopodite and exopodite are attached. The primitive state of the protopodite of the biramous limb is considered to be unsegmented with a solitary endite. Numerous arthopod taxa possess a non-segmented protopodite with one endite (see fig.1.2D). Examples of single endites on undivided protopodites include all post-antennal limbs of trilobites, the prosomal limbs of horse shoe crabs (Xiphosurans), the pedipalps of

[1] These monopodial limbs are posterior to antennae that are of uncertain homology, but the base of these antennae may be homologous to a structure present in all euarthropods called the Labrum.

spiders, and the first two legs of scorpions and this is considered to be the primitive state.

Mandibulate arthropod appendages that have endites include the mandible, naupliar antennae of crustaceans, the post-mandibular limbs of cephalocarid and branchiopod crustaceans, and the maxillae of hexapods, chilopods and symphylans and crown group crustaceans. There are also many appendages that possess multiple endites on each segment, for example in the Cambrian fossil crustacean *Rehbachiella kinnekullensis* there are up to nine endites on the unsegmented protopodite of the trunk limbs and four present on the maxilla (Walossek 1993). There are up to 6 endites present on extant branchiopods. There are usually two endites present on the maxilla of Hexapods called the lacinia and galea (Boxshall, 2004).

Mandibulate arthropod protopodites are often segmented which indicates that the protopodite has added segments to the primitive protopodite. It also means that the endites of chelicerates and trilobites are not homologous to the endites of the mandibulate arthropods. In order to conclude about the serial homology of the mandible to other appendages of mandibulate arthropods, it is necessary to identify which segment the mandibular gnathal edge is attached to, so that it can be compared to the segments of other appendages.

Evolution of the biramous limb into all arthropod appendages.

A characteristic of arthropods is the possession of a wide range of specialized limbs and appendages adapted for a plethora of different functions. The arthropod body plan with its basic composition of a series of repeating segments, each bearing a pair of appendages which can evolve as a module separate from other segments, has most likely facilitated the evolution of this group to fit such a wide variety of habitats (Williams and Nagy, 2001; Giorgianni and Patel, 2005).

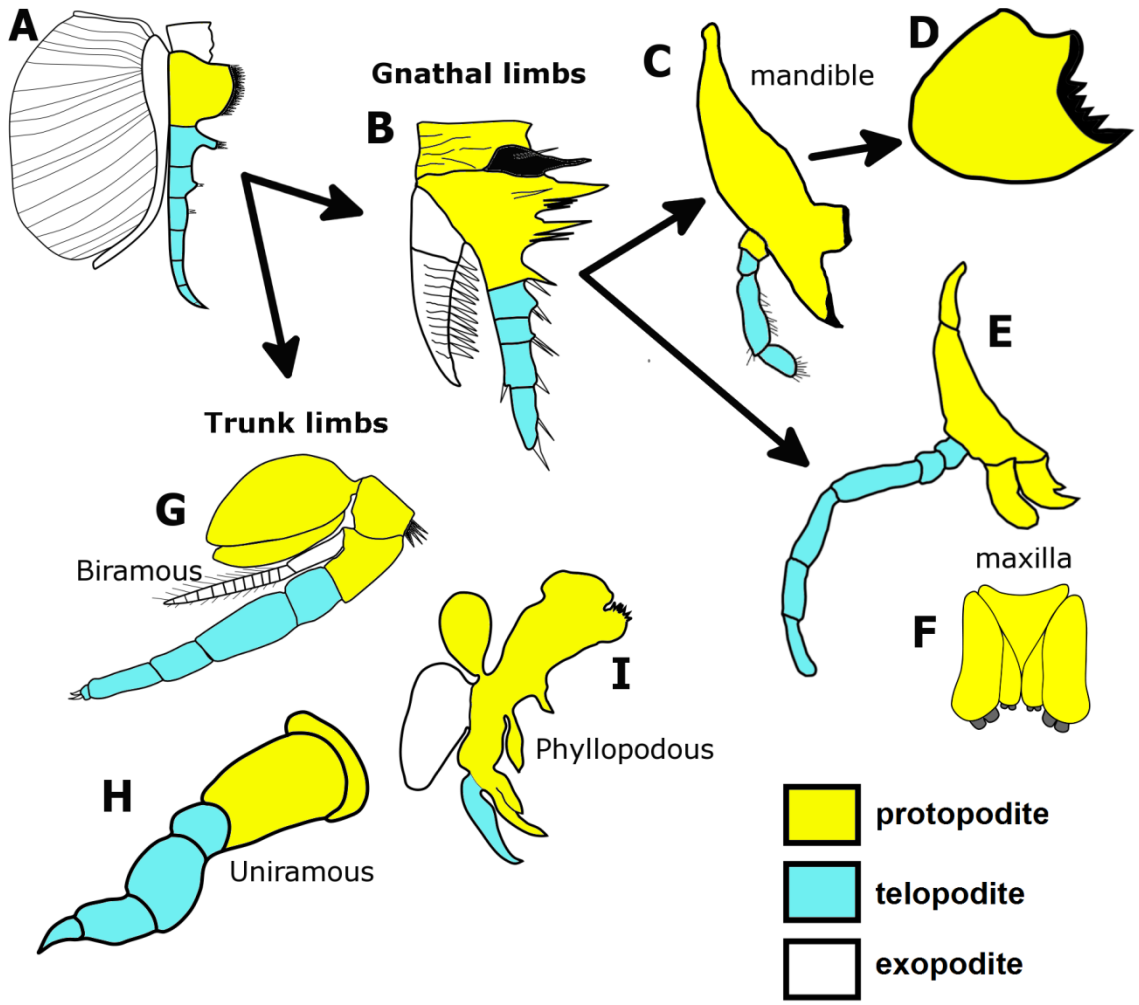

Fig.1.3. Evolution of post-antennal arthropod appendages from the ancestral biramous limb. The telopodite is highlighted blue, the protopodite is highlighted in yellow, the exopodite is highlighted white. The ancestral biramous limb (A,B) evolved into the gnathal appendages such as the mandible with palp (C), mandible without a palp (D), maxilla (E), gnathochilarium (F) or trunk appendages such as the biramous limb (G), uniramous limb (H) and phyllopodous limb (I). The proximal endite from which the mandibular gnathal edge may have evolved (present in some Cambrian fossils such as *Martinssonia*) is highlighted in black in (B).

All post-antennal appendages such as the mouthparts and trunk limbs of Euarthropoda evolved from the ancestral biramous limb (see fig.1.3). Appendages near the anterior of the organism are often adapted to form sensory appendages and mouthparts such as the mandibles, maxillae, chelicerae, labial and second antenna (fig.1.3B-F). Sensory specialization is evident in the palps, endites, and the abundance of sensory organs on these appendages. Evolution of these appendages is often associated with incorporation of their segments into head 'tagma' (an aggregation of segments into a distinct unit), for example into the heads of mandibulates.

Appendages of the trunk, by contrast, are often adapted to particular modes of locomotion. For example, uniramous limbs have evolved in numerous terrestial arthropods such as the myriapods, insects and isopods (woodlice) (fig. 1.3H). In these

lineages, the expodite has been lost (likely due to convergent evolution). There are no biramous limbs present in myriapods or hexapods. The biramous limb is often retained in taxa that reside in aquatic environments (See fig.1.3G.). One form of biramous limb - phyllopodous limbs - is paddle-like and has evolved for swimming in taxa like the branchiopods (See fig.1.3I). Appendages that are near the posterior of the animal are often lost, for example the abdomen of insects, and the opisthosoma of chelicerates typically lack appendages (Boxshall, 2004; Angelini and Kaufman, 2005; Giorgianni and Patel, 2005).

Evolution of the biramous limb into the mandible.

The mandible is an evolutionary novelty that permitted numerous additional means of capturing prey or processing food using a biting or cutting tooth (*pars incisiva*), and a grinding or crushing molar process (*pars molaris*). The mandible, like all post-antennulary appendages, evolved from a biramous limb with endites present on the base of the limb. A possible scheme of mandible evolution is illustrated in fig.1.7. The mandible gnathal edge has probably evolved from a biramous limb by modification of the proximal endite on the protopodite (fig. 1.7A,B), reduction of the size of the telopodite and exopodite (fig. 1.7C,F) and changes in protopodite structure resulting from adaptations to functioning as a biting/chewing mouthpart. Numerous lineages have lost the exopodite (fig.1.7F) and in many cases the telopodite too (fig.1.7D,E,G,H).

One structure that the gnathal edge may have evolved from is the proximal endite as seen on the protopodite of extinct species such as *Martinssonia and Henningsmoenicaris* that is larger and more developed than other endites (Muller and Waloszek, 1986; Boxshall, 2004; Waloszek et al., 2007; Edgecombe, 2010; Rota-Stabelli et al., 2011). The proximal endite of *Martinssonia* is shown in fig. 1.6C,D and 1.7A**.**

1.3 Mandible diversity

The mandible is the anterior-most of the gnathal appendages (mandible, maxilla and labial appendages) in the gnathocephalon (see fig. 1.1C), the posterior head region of mandibulates. The labrum forms an upper lip that, together with the mandibles and hypopharynx, forms a chewing chamber (Bucher and Wimmer, 2005; Scholtz and Edgecombe, 2006; Edgecombe, 2010). The pair of mandibular appendages

flank the mouth-opening and sit underneath the labrum which forms the roof of the mouth. The similar arrangement of the mandible relative to other appendages on homologous segments suggests that the mandible is homologous in all mandibulates (Wägele, 1993; Bitsch, 1994; Edgecombe, 2010). The mandible is followed by more posterior manipulative and sensory appendages called maxillary appendages or maxillae. Some maxillary appendages have fused at the ventral midline, for example the gnathochilarium of diplopods and the labial appendages of hexapods.

The primitive function of the mandible is a biting chewing appendage that can also be used to manipulate food. The biting edge consists of two parts that are common to the majority of mandibles, an incisor process (the *pars incisiva*) and a molar process (the *pars molaris*), which are shown in fig.1.1. This type of mandible is present in the majority of taxa within the Mandibulata. All millipedes and centipedes and the majority of crustaceans (if not all) are in possession of this type of mandible. The insects display considerably more diverse functional adaptations of the mandible and the gnathal appendages in general than other members of Mandibulata.

While the biting/chewing mandible still remains in the majority of insect taxa[2], such as the orders Orthoptera, Coleoptera, Hymenoptera and numerous basal orders relative to the Holometabola, it has adapted to numerous functions in different taxa. The maxillary and labial appendages of the ants (Formicidae) are reduced, and instead the mandibles are used for numerous functions that include defensive, manipulatory, predatory functions and for lifting and carrying objects. The mandibles of male stag beetles (family Lucanidae) are greatly enlarged and used in male combat. The leaf-feeding scarab beetle *Popillia japonica* has an enlarged molar process and reduced incisor process on the mandible. The galea enditic lobe of the maxilla has become dentate and functions to cut leaves instead of the mandible whilst the mandible molar surface has evolved into a masticatory organ. In some leaf-mining lepidopteran larvae the molar process has evolved to become saw-like as opposed to a crushing/grinding tooth surface. Outward facing mandibles are present in the parasitic elephant louse *Haematomyzus* and the leaf mining saw fly *Phyllotoma aceris* (Tenthredinidae). The adult dung beetle *Onthophagus* feeds on soft, fluid portion of dung and no longer requires mandibles to chew and the mandibles of this species have evolved to function

[2] Two of the most diverse insect orders, the Hymenoptera and Coleoptera, possess biting/chewing mandibles.

in sifting and filter feeding. Other beetle species, for example the tiger beetles (Cicindelidae) that digest food extra-orally have lost the molar process (Snodgrass, 1950; Holldobler and Wilson, 1994; Hughes and Kaufman, 2000; Angelini and Kaufman, 2005; Grimaldi and Engel, 2005; Simonnet and Moczek, 2011).

The mouthparts of several insect orders have been modified even further from typical mandibulate mouthparts into piercing or sucking mouthparts. These include numerous members of the superorder Paraneoptera such as Hemiptera (a diverse order including aphids, cicadas, leafhoppers, planthoppers, and shield bugs), Thysanoptera (thrips) and Phthiraptera (lice), as well as the Lepidoptera (butterflies and moths), Siphonaptera (fleas) and numerous dipteran families (Grimaldi and Engel, 2005). The mandibles in these lineages have undergone more drastic modifications, or reduction or loss of the entire appendage itself. The mandibles are lost and the maxillae are highly reduced in cyclorrhaphous dipterans like *Drosophila*. Instead, the proboscis is derived from the labial appendage which forms the sponge-like labella. Profound modifications have occurred in other lineages, for example, mandibles are modified in some blood-sucking dipterans (like mosquitos from the family Culicidae) to form accessory lancets that aid in puncturing skin. The stylet of Hemipterans such as *Oncopeltus* is derived from the mandibular and maxillary appendages and modified into a tube.

Condyles

The primitive mandible has one dorsal attachment point called a condyle or articulation. The mandible is suspended like a pendant from this articulation. This pendant mandible with one articulation, known as a monocondylic mandible, is plesiomophic and is present in numerous crustaceans and archaeognathan hexapods (see fig.1.4B). The maxilla, is also a monocodylic appendage (fig. 1.4D). There are variations in mandibular structure, for example in the numbers of articulations or attachment points of the mandibles.

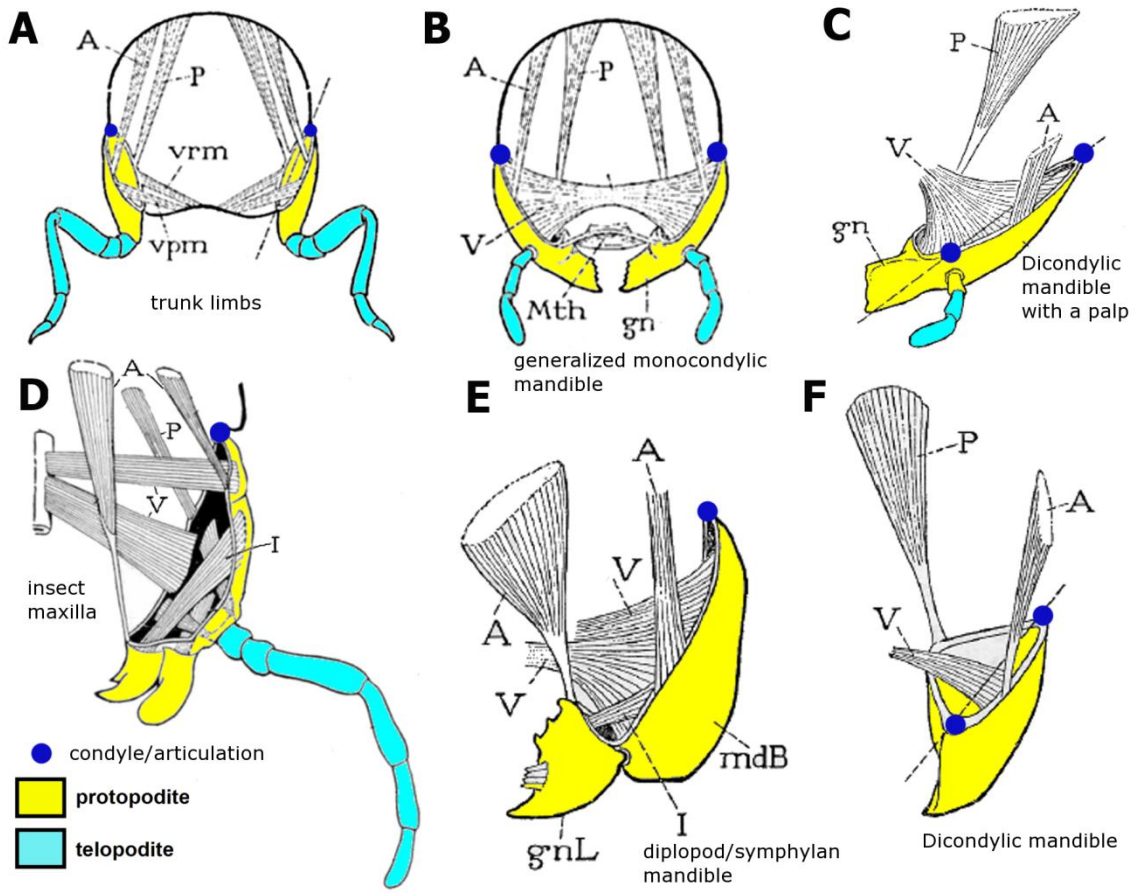

Fig.1.4. Examples of mandibular articulations and musculature compared to an insect maxilla and leg. Figure is adapted from Snodgrass (1935, 1950). Condyles/articulations are labelled as blue dots. The axis of rotation is indicated by a dotted line between these two articulations. The telopodite is highlighted blue, the protopodite is highlighted in yellow. (A) Generalised protopodite musculature of an arthropod leg appendage. The dorsal premotor (A) and dorsal remotor (P) muscles of the leg are homologous to the anterior rotator (A) and the posterior rotator (P) muscles of the mandible. Muscles are attached to the dorsal body cuticle (the tergum). The ventral promotor (vpm) and ventral remotor (vrm) are homologous to the ventral muscles (V) of the mandible. These muscles are attached to the ventral body cuticle (the sternum). (B) Generalized pendant monocondylic mandible present in Archaeognatha and numerous crustacean taxa. The ventral adductor is the main muscle used to shut the mandible jaws in front of the mouth (Mth), the muscles are attached to each other with a ligament. The A and P muscles are attached to the dorsal head capsule. (C) A generalized decapod mandible with two articulations and a gnathal edge in line with the axis of rotation present in the majority of Malacostraca and Chilopoda. (D) Generalized insect maxilla. The maxilla is monocondylic, with notable similarities of the A, P, and V muscles to both the leg and mandible. The lacinia is attached by a muscle to the stipes segment. (E) Generalized diplopod/symphylan mandible with an independently movable gnathal lobe (gnL) connected to the mandible base (mdB) by a muscle (I). (F) Generalized dicondylic mandible with perpendicular orientated gnathobase present in Isopoda, Amphipoda, Lepismatidae and Pterygota. The posterior rotator is greatly enlarged and has taken over the function of closing the mandible.

Some arthropods possess mandibles with two articulation points with the gnathal edge orientated in line with the axis of rotation (fig.1.4C) or perpendicular to the axis of rotation (fig.1.4F). This structural arrangement is particularly well adapted to the function of biting and chewing as the axis of rotation enables the gnathal edges of the mandible to open and close more forcefully and is present in the some crustaceans (Isopoda and Amphipoda). It is also present in the majority of insects. These insects form a clade called the Dicondylia which includes the Ephemeroptera and Zygentoma (Engel and Grimaldi, 2004). The mandible of Dicondylia is doubly articulated with two attachment points (condyles) and a perpendicularly orientated gnathobase. These doubly articulated mandibles of some crustaceans and insects are convergent structures.

The mandible gnathal lobe in myriapods

The myriapod mandible has the gnathal edge present on either a flexible or movable gnathal lobe that is independently musculated (see fig.1.4E). This movable lobe is held against the mandible base. Chilopod (centipede) mandibles possess a flexible gnathal lobe, whilst the Diplopoda (millipedes) possess mandibles with a gnathal lobe that is clearly separated (see fig.1.4E, fig.1.7D,E and fig.1.9C). The diplopod mandible consists of two segments, a cardo and stipes (see fig.1.9C).

Mandibular palps

One of the most obvious indications that the mandible has evolved from the base of the leg is the presence of a palp (corresponding to the long articulated leg) on some crustacean mandibles (see fig.1.1F and fig.1.4B,C). The mandibles of ostracod crustaceans are the most leg-like, and have also lost the characteristic gnathal edge (Snodgrass, 1950; Boxshall, 2004; Richter and Kornicker, 2006). The primitive mandible would have possessed two palps, an exopodite derived palp and a telopodite derived palp as is present in Cambrian arthropods (see fig.1.7B,C). Loss of these two palps has occurred frequently throughout Mandibulata.

The two rami of biramous mandibles, which represents the ancestral condition, have been lost in almost all mandibulate lineages. Biramous mandibular appendages are present in crustacean nauplius larvae, and in ostracod mandibles. Mandibles with a single telopodite-derived palp are the most common form of mandible with a palp and

are exclusively present in crustacean taxa such as branchiopods, cephalocarids and malacostracans (Snodgrass, 1950).

The telopodite derived palp has been lost independently from the mandibular appendage in several lineages of mandibulates (see fig.1.3D). The palp has been lost in all hexapods and myriapods. Loss of the palp therefore occurred in the stem lineage to each of these groups. Numerous crustacean taxa have also lost the telopodite derived palp such as isopod crustaceans (woodlice). The loss of the mandibular palp in terrestial taxa is a possible example of convergent evolution to a terrestial habitat adaptation.

Homology of the arthropod mandible

In spite of all the diversity of mandibular structures outlined above, the mandible of insects, crustaceans and myriapods is considered to be a homologous structure that had a single origin in the ancestor to the mandibulate arthropods. Comparison of the musculature of the mandible to the base of a leg (the coxa) reveals similarities in structure that suggest that the mandible has evolved from a leg (see fig.1.4). According to Snodgrass, there are obvious homologies between different muscles of more primitive mandibles with those of typical trunk appendages (Snodgrass, 1950). In the coxa of generalized legs, there are two pairs of muscles attached to the coxa, a ventral pair and a dorsal pair (fig.1.4A). In the generalized primitive mandible, there are homologous muscles (shown in fig.1.4B). Snodgrass hypothesized that the mandibulate arthropods constituted a monophyletic group called the Mandibulata.

Manton hypothesized *contra* Snodgrass that the mandible of myriapods and hexapods was telognathic, that is to say, the gnathal edge was present on the telopodite (rather than protopodite) of the mandible. In place of Mandibulata, Manton constructed the clade Uniramia that included myriapods, hexapods and onychophorans (Manton, 1964; Manton, 1977). This view has been thoroughly disproved by evidence from molecular development that the mandible of hexapods and myriapods is in fact made from the base of the limb (gnathobasic).

The mandibular gnathal edge is a homologous structure

The defining character of the mandible that distinguishes it from other appendages is the gnathal edge (see fig.1.1A,B,D,E). The gnathal edge of the

mandibular appendage of myriapods, crustaceans and hexapods is considered homologous by Edgecombe *et al.* (Edgecombe et al., 2003). This structure originated once in the ancestor to mandibulate arthropods and is evidence of the monophyly of this group (Snodgrass, 1950; Kraus, 2001; Edgecombe et al., 2003). The gnathal edge is often made of three parts, the incisor and molar processes and a small moveable dentate plate structure called the *lacinia mobilis.* The authors conclude from comparative morphology of mandibles across Mandibulata that the incisor and molar processes may be homologous. The incisor process is the distal-most structure of the gnathal edge and is dentate, made up of tooth-like structures. The hypothesized primitive structure of the molar process is made up of a series of rows of spines. In many pancrustacean mandibles, this basal configuration of rows of spines is covered with scaly transverse ridges, which is interpreted to be a derived state.

Morphological characters in support of Mandibulata

Mandibulata[3] as a group was originally hypothesized on morphological grounds and there are numerous morphological characters in support of Mandibulata, including the structure of the mandibular gnathal edge, the particular mandibulate arrangement of segments in the gnathocephalon (see fig.1.1C), along with a whole suite of neurological and morphological characters (Snodgrass, 1938; Snodgrass, 1950; Edgecombe et al., 2003; Edgecombe, 2010).

[3] The original Mandibulata hypothesis included the Atelocerata plus Crustacea rather than Pancrustacea/Tetraconata plus Myriapoda.

1.4 Arthropod Phylogeny

One method to determine the evolutionary history of a morphological character, such as the mandible, which displays both significant diversity and elements of similarity that could represent homology or convergent evolution, is to map developmental and morphological characters onto a robust phylogenetic tree. By evaluating the most parsimonious explanation of the distribution of character states, they can be described as either novel or ancestral characters (Akam, 2000; Telford and Budd, 2003; Jenner, 2006). In order to do this, it is necessary to have a robust phylogeny on which to place these characters.

Despite the attention given to the phylum and the years of research devoted to understanding their evolutionary history, the origin of the arthropods and the phylogenetic relationships between the different subphyla are still uncertain.

Articulata and Atelocerata: two early victims of the new molecular phylogeny.

Molecular phylogenies have overturned numerous phylogenetic hypotheses based on more traditional cladistic analyses of morphology in the last two decades. One of the first victims was the Articulata, which united all panarthropods together with segmented annelids. The Ecdysozoa has replaced it. Ecdysozoa includes panarthropods and the Cycloneuralia (Nematoda, Nematomorpha, Priapulida, Kinoryncha and Loricifera). The Ecdysozoa has strong support from molecular based phylogenies (Aguinaldo et al., 1997; Telford et al., 2008; Rota-Stabelli et al., 2010). The Ecdysozoans are characterized by their ability to moult (ecdysis).

Monophyly of the Arthropoda is strongly supported by molecular and morphological data (Mallatt et al., 2004; Mallatt and Giribet, 2006; Bourlat et al., 2008; Dunn et al., 2008; Edgecombe, 2010). The extant sister group to the Euarthropoda, the true arthropods, is considered to be either the Onychophora or the Tardigrada (Rota-Stabelli et al., 2010). Onychophora is more commonly recognised as sister group to the arthropods and has some support from molecular phylogenies (Roeding et al., 2007; Dunn et al., 2008). A more distant sister group to the Arthropoda are the priapulids and nematodes (Rota-Stabelli et al., 2010).

Tardigrada, the water bears, have been included with Euarthropoda to form the Tactopoda. Onychophora, the velvet worms, do not possess jointed appendages or a

hardened cuticle. Arthropods and Tardigrades share a cuticle and jointed legs, although the segmented leg appendages of Tardigrades are telescopic in nature rather than resembling arthropod segmented appendages (Edgecombe, 2010).

Recent molecular phylogenetic analyses as well as morphological analyses have placed the Hexapods with crustaceans to form a monophyletic clade called the Pancrustacea/Tetraconata (Friedrich and Tautz, 1995; Telford and Thomas, 1995; Boore et al., 1998; Cook et al., 2001). The Hexapoda originated from within the crustaceans, and the crustaceans are therefore paraphyletic. Previous classification schemes had united myriapods, hexapods and onychophorans in the group Uniramia (Manton, 1977), or more traditionally grouped myriapods and hexapods together in the Atelocerata/Tracheata (Snodgrass, 1935) whilst excluding the crustaceans.

Sister group relationships within Arthropoda

The monophyly of the Hexapods, Myriapods and Chelicerates is widely supported, as is the paraphyly of the crustaceans (monophyly of Pancrustacea). Much of the difficulty of constructing an arthropod phylogenetic tree concerns the relationships between these taxa (Edgecombe, 2010). There is no consensus for the crustacean sister group to the insects (Edgecombe, 2010; Jenner, 2010).

Sister group to the Pancrustacea: Mandibulata vs. Myriochelata

Accepting the validity of the Pancrustacea, more recent controversies lie in the position of the myriapods in relation to other arthropod groups, namely the Pancrustacea or Chelicerata (Caravas and Friedrich, 2010; Edgecombe, 2010). If myriapods are grouped with Pancrustacea they form the monophyletic group the Mandibulata. If Myriapods are sister group to the chelicerates then they comprise the monophyletic group the Myriochelata or Paradoxopoda. These two competing phylogenetic hypotheses are illustrated in fig. 1.5**.**

Acceptance of Mandibulata or Myriochelata has important implications regarding the evolution of the mandible. Acceptance of Mandibulata is compatible with, or even suggestive of, the homology of the mandible across mandibulates. If Myriochelata is valid, this would strongly imply that the mandible has evolved independently in the lineages that lead to the Myriapoda and the Pancrustacea (Mayer and Whitington, 2009) or has reversed into a leg in the chelicerate lineage. If it is found that the mandibular patterning mechanisms (the focus of the present study) are

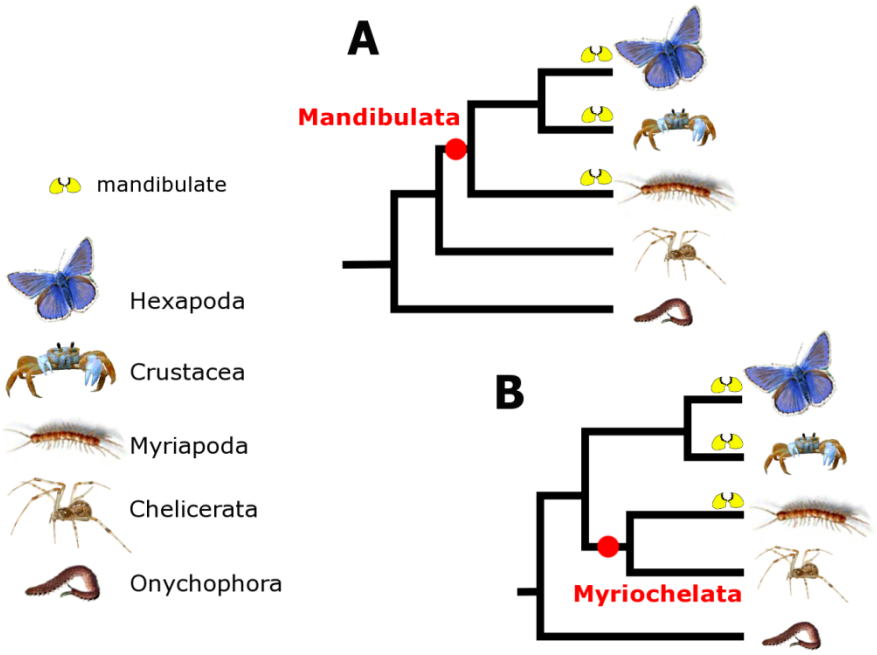

Fig.1.5. Two competing Arthropod phylogenetic relationships, the Mandibulata and Myriochelata hypotheses. Arthropoda are monophyletic and include the extant groups: Hexapoda, Crustacea, Myriapoda and Chelicerata. Onychophora are sister taxa to the Arthropoda. Hexapoda, Crustacea and Myriapoda are mandibulate. The difference between the two hypotheses concerns the position of the Myriapoda. (A) The Mandibulata includes the Myriapoda as sister group to the Hexapoda and Crustacea. (B) The Myriochelata includes the Myriapoda as sister group to the Chelicerata.

conserved between diverse lineages of Mandibulates, this would provide additional support for the monophyly of mandibulates.

Molecular phylogenies of 28S and 18S ribosomal RNA have tended to support Myriochelata over Mandibulata (Friedrich and Tautz, 1995; Giribet et al., 1996; Mallatt et al., 2004). Phylogenies based on single nuclear genes such as hemocyanin (Kusche and Burmester, 2001) and Hox genes (Cook et al., 2001) have also favoured Myriochelata. However, these phylogenies based on alignment of single genes are highly susceptible to stochastic error artifacts due to the small size of the datasets.

Studies have therefore sought to overcome the limitations of single gene phylogenies by increasing the sample size of the datasets. Phylogenies which are made from concatenated mitochondrial genes support Myriochelata over Mandibulata (Hwang et al., 2001). It has been shown recently that the choice of outgroup will affect support for either Mandibulata or Myriochelata (Rota-Stabelli and Telford, 2008). Selecting outgroups based upon similarity of nucleotide composition results in phylogenies that favour Mandibulata. An outgroup that is considered more appropriate is the Priapulida. Less appropriate outgroups are the Nematoda and the

more closely related Onychophora. Combination of mitochondrial genes and nuclear genes to create a phylogeny have produced mixed results, supporting either Myriochelata (Pisani et al., 2004) or Mandibulata (Bourlat et al., 2008).

With the advance of genome sequencing technologies, phylogenetic studies have started to incorporate numerous genetic loci. Two notable early examples of phylogenomic studies strongly favoured Myriochelata (Dunn et al., 2008; Philippe et al., 2009). These studies were concerned with the phylogeny of the Metazoa and, as a result, sampling within the Arthropoda was limited. More recent phylogenomic studies that have focused on the relationships of Arthropods, has favoured Mandibulata (Regier et al., 2008; Regier et al., 2010; Rota-Stabelli et al., 2011).

There is support for Mandibulata from the presence of characters of rare genomic change, such as micro RNAs (miRNA). miRNA sequences are highly conserved, and are rarely lost from the genome once acquired (Hertel et al., 2006). The presence of particular miRNAs, miR-965 and miR282, in all sampled mandibulates but not chelicerates supports Mandibulata (Rota-Stabelli et al., 2011).

It is clear then that some molecular phylogenies, particularly earlier studies, have favoured the Myriochelata, a clade which draws little support from morphological data. More recent phylogenies that include larger datasets, reduce the presence of long branch attraction, use appropriate models and choice of outgroup and incorporate rare phylogenetic changes are favouring Mandibulata over Myriochelata (Regier et al., 2008; Rota-Stabelli and Telford, 2008; Regier et al., 2010; Rota-Stabelli et al., 2011). It is apparent that the internal branch lengths leading to either group are short, indicating that presence of a poor phylogenetic signal which is difficult to resolve (Rota-Stabelli and Telford, 2008).

In view of the recent molecular phylogenies in support of Myriochelata, there has been some attempt to identify morphological characters in support of Myriochelata. For example, there are similarities in the development of the nervous system and neurogenesis between spiders and myriapods (Stollewerk and Chipman, 2006; Mayer and Whitington, 2009).

Considering the strong level of support for Mandibulata from morphological data, and the increasing level of support from more recent molecular phylogenetic data, Mandibulata has more overall support than Myriochelata. As more recent phylogenies with larger datasets and more consideration to sources of potential bias

favour Mandibulata, on balance, Mandibulata must be considered as the more favourable hypothesis.

1.5 Arthropod Fossil Record in the Cambrian

An understanding of the evolution of the mandible requires an examination of the fossil record, to show us when the mandible was likely to have evolved and what kind of appendage it evolved from. Crown group arthropods evolved in the Cambrian. Supposed fossil representatives of two major extant groups of arthropods, chelicerates and crustaceans, are present during this period.

The fossil record for arthropods during the Cambrian is rather good both in terms of level of preservation (complete specimens, embryos and larval stages) and the variety of organisms found. Numerous fossil arthropods have been discovered at several locations representing the lower Cambrian such as the Chengjiang (Chen, 2009) and the middle Cambrian as represented by the Burgess Shale fauna (505 million years ago). Numerous small, soft-bodied fossils are preserved from the lower and upper Cambrian, known as Orsten fauna (Waloszek and Maas, 2005; Budd and Telford, 2009). Orsten fossils are very small, preserved specimens - typically larval forms less than two millimetres in size (Maas et al., 2006).

Despite the abundance of Cambrian arthropod fossils, the assignment of these fossils to particular clades of arthropods has not been easy, primarily because of the presence of stem lineage arthropods which display an enormous diversity of forms. This is in contrast to extant arthropods, which whilst diverse, are more easily organised into groups that possess easily distinguishable body tagma and synapomorphies. The variety of Cambrian stem lineage forms is likely to represent a collection of paraphyletic groups which differ from the crown group, lacking significant apomorphies present in the crown group (Grimaldi and Engel, 2005). Arranging these stem lineage forms which share some characters but not others of the crown group makes assigning taxonomic relationships a complex task. However, increasingly palaeontologists are arriving at a consensus (Budd and Telford, 2009).

The earliest fossil example of a true insect (Ectognatha) is a probably pterygote mandible, *Rhyniognatha hirsti* that was discovered in the Rhynie chert (dating to about 396-407Mya) which points to the possible presence of wingless insects present on land along with myriapods and chelicerates during the Silurian. The earliest likely hexapod

fossil is *Rhyniella praecursor* that dates from the same period (Entognatha, Collembola) (Whalley and Jarzembowski, 1981; Engel and Grimaldi, 2004).

Cambrian crustaceamorph fossils

Identification of an ancestor to all mandibulate arthropods could help in characterizing the primitive mandible. It could provide evidence for particular characters present on the primitive mandible such as the number of segments present on the protopodite, the presence of a gnathal edge with incisor and molar processes, the number of palps and location of the gnathal edge on a particular segment.

Currently there is disagreement on the placing of particular Cambrian arthropods that are obviously ancestral to the Pancrustacea but share numerous characters with crustaceans. These fossils have been described as crustaceamorphs due to their resemblance to crustaceans (Waloszek et al., 2007). One such example is *Martinssonia elongata* (see fig.1.6).

If either Myriochelata or the Mandibulata hypothesis is true, one challenging problem to explain is the fact that there is no obvious stem lineage myriapod fossil for one hundred million years after the first appearance of crustaceans in the Cambrian. The lack of any fossils of a particular lineage when it is expected that there should be is known as a ghost lineage. The earliest myriapods that appear in the fossil record in the Silurian about 420 million years ago are *Cowiedesmus eroticopodis* and *Archaedesmus macnicoli* from Scotland (Wilson, 2004; Shear and Edgecombe, 2010).

A myriapod ancestor should be present in the Cambrian as there are fossil representatives of crown group crustacean taxa present such as branchiopods like *Rehbachiella kinekullensis* (Olesen, 2007; Waloszek et al., 2007)*, Castracollis baltica* (Olesen, 2007) and the maxillopods *Bredocaris* and *Dala peilertae* (Muller, 1983).

It is possible that myriapod fossils were not abundant due to the rarity of myriapod species or the myriapod habitat not being conducive to fossil preservation (Shear and Edgecombe, 2010). Another explanation of this ghost lineage is that stem lineage myriapods were crustaceamorphs, their body plans sharing a significant number of crustacean characters which have been lost in the stem lineage to the myriapods. Some primitive characters have certainly been lost in myriapods such as the palps on the mandibles, the exopodites on all limbs, and the second antennae.

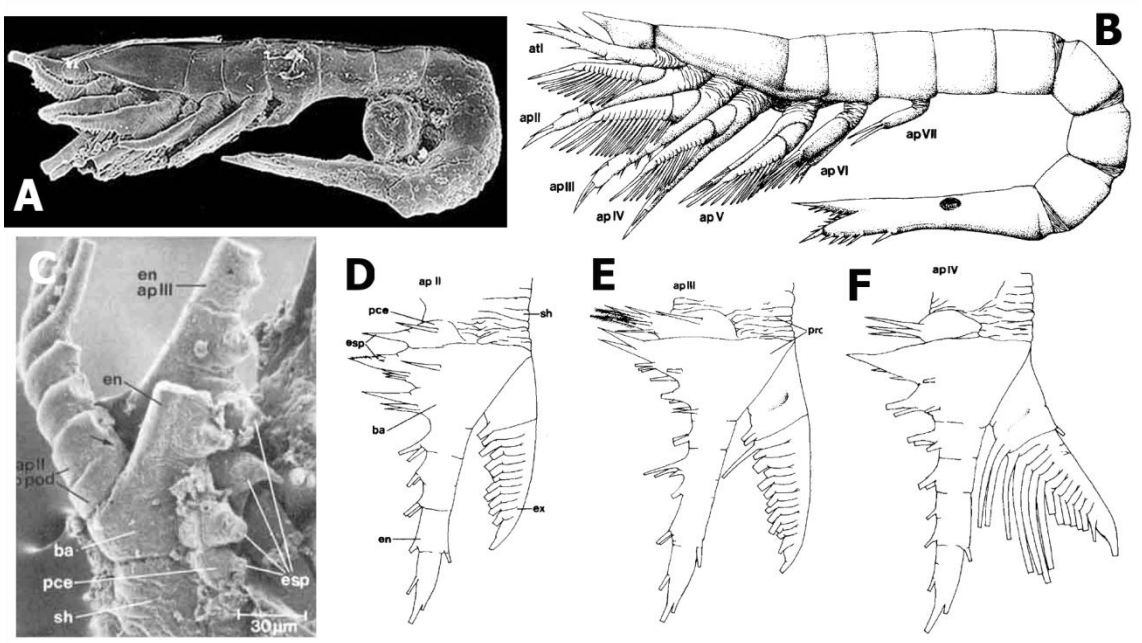

Fig.1.6. The crustaceamorph *Martinssonia elongata*, a possible stem lineage representative of Mandibulata. figure is adapted from Muller and Waloszek (1986). (A) SEM of *Martinssonia* larva. Anterior is to the left, dorsal is up. (B) Schematic of (A). (C) SEM of the second appendage and third appendage. (D) Schematic of the second appendage, the first post-antennulary appendage homologous to the second antenna of Crustaceans. (E) Schematic of the third appendage, the second post-antennulary appendage homologous to the mandible. (F) the fourth appendage, third post-antennal appendage homologous to the first maxilla of mandibulates. *Martinssonia* shows features of a possible stem lineage mandibulate such as relatively undifferentiated serially homology biramous limbs. There is an enlarged proximal part with endites on the medial side. The second post-antennal appendage (homologous to the mandibular segment) does not possess a characteristic mandibulate gnathal edge, which is required to define it as a true mandibulate. The postantennal limbs have three endites, the most proximal of which are more developed and form a moveable plate-like structure. There is no typical mandibular gnathal edge present. The proximal endite on the second postantennal limb, homologous to the mandibular limb, is more developed than the endites of other limbs and could represent the developing mandibular endite. Abbreviations are as follows: telopodite (en), exopodite (ex), protopodite (pro) basis (ba), shaft (sh), proximal endite (pce), endite spines (esp), antenna (atl), second (apII), third (apIII), fourth (apIV), fifth (apV), sixth (apVI), and seventh appendages (apVII).

These losses have also occurred in the hexapod lineage, leading to the idea that these may be convergent losses due to adaptations to a terrestial environment.

Therefore, the apparent lack of stem lineage mandibulate or myriapod fossils may actually be due to mis-interpretation of Cambrian crustaceamorph fossils. As crustaceamorph fossils share most characters with crustaceans there is a bias favouring the placing of these crustaceamorph fossils as crustaceans. Some of them may in fact be stem lineage mandibulates or myriapods. Identification of a myriapod fossil could help in re-constructing the primitive mandible by comparing the mandible present on ancestral myriapods to the mandibles of ancestral crustacean species.

Stem lineage Mandibulata

If Mandibulata is accepted with the mandibular gnathal edge as a synapomorphic character of this group, a well-defined mandible (complete with incisor and molar processes) should be present in stem lineage mandibulates.[4] (Kraus, 2001; Edgecombe et al., 2003). If crustaceamorph fossils are lacking true mandibular appendages, then they cannot be either crustaceans or crown group mandibulates. One crustaceamorph fossil that could, however, be interpreted as a member of the stem lineage leading to Mandibulata[5] is *Martinssonia elongata* (Muller and Waloszek, 1986) (see fig. 1.6).

The proximal endite on the second postantennal limb of *Martinssonia*, homologous to the mandibular limb, is thought to represent the developing mandibular endite in the stem lineage to Mandibulata (Edgecombe, 2010; Haug *et al.,* 2010; Rota-Stabelli et al., 2011). The appendages on the segments homologous to the mandibular and maxillary segments, the second (see fig. 1.6E) and third (see fig. 1.6F) post-antenullary appendages of *Martinssonia*, are similar to one another. A reasonable hypothesis is that the mandible evolved through a biramous maxilla like appendage. The primitive maxilla and maxilla-like precursor of the mandible would have probably shared similar developmental pathways as they are serially homologous appendages. To understand the evolution of the mandible may therefore be helped by an understanding of maxilla development, and subsequent understanding of how the mandible is differentiated from a maxillary appendage.

[4] Contrary to this view, Waloszek et al. on the C.O.R.E. website have stated: "a mandible was NOT present in the beginning of the evolution of Crustacea, so any names referring to a mandible, such as Mandibulata, are misleading or wrong" http://www.core-orsten-research.de/

[5] Other possible stem lineage mandibulates include: *Oelandicaris, Cambrocaris, Cambropachycope, Goticaris, Henningsmoenicaris scutula, Tanazios dokeron, Apankura machu* and Phospatocopida (Siveter *et al,* 2001; Vaccari *et al.* 2004; Siveter *et al.* 2007; Zhang *et al.* 2007; Edgecombe, 2009)

Chelicerate evolution and stem lineage fossils

The only extant clade of non-mandibulate arthropods is the Chelicerata, comprised of arachnids (spiders and scorpions), xiphosurans (horseshoe crabs) and pycnogonids (sea spiders).

Identification of the stem lineage of Chelicerates is also problematic due to fossils lacking characters only present in crown group chelicerates (autapomorphies). There are, however, numerous Cambrian fossils that are hypothesized to be part of the stem lineage to Chelicerata.[6] These taxa are characterized by the presence of biramous limbs, like stem lineage arthropods as well as possessing similarities to two characters which are diagnostic for members of the chelicerate crown group: the chelicerae (pincer-like appendages that may have evolved from antennal appendages) and the prosoma associated with six pairs of appendages. The presence of structures that may represent precursors of these two characters on particular Cambrian fossils, such as the megacheiran great appendage (which has been interpreted as a precursor to the chelicerae), favours their placement on the stem lineage to Chelicerata.

It has been shown that the first leg segment of extant Chelicerates is homologous to the mandibular segment of Mandibulata based upon the anterior expression of Hox genes such as *Dfd* (Damen et al., 1998; Telford and Thomas, 1998b). Therefore, as the mandible has evolved from a leg appendage, studying the development of the first leg appendage of chelicerates (particularly less derived forms present in the Xiphosura) could aid our understanding of mandible evolution.

Understanding of non-mandibulate appendages present in chelicerates and trilobites is important to identify the ancestral condition of the mandibular appendage. It is also important for determining homology of the protopodite segments and endites between mandibulates and non-mandibulate groups.

[6] For example possible stem lineage chelicerates include *Sidneiya, Sanctacaris vacata,* and *Aglaspis spinifer* Boxshall, G. A. (2004) 'The evolution of arthropod limbs', *Biol Rev Camb Philos Soc* 79(2): 253-300, Budd, G. E. and Telford, M. J. (2009) 'The origin and evolution of arthropods', *Nature* 457(7231): 812-7, Chen, J.-Y. (2009) 'The sudden appearance of diverse animal body plansduring the Cambrian explosion', *Int J Dev Biol* 53(5-6): 733-51.. Also, the great appendage arthropods (megacheirans) are often considered as a stem group of the lineage leading to Chelicerata (designated Prochelicerata by Chen), for example, *Haikoucaris, Fortiforceps, Leanchoilia, Yohoia*.

1.6 Serial homology of the mandible and maxilla

In this section I will present a hypothetical scheme of mandible (fig.1.7) and maxilla (fig.1.8) evolution from a primitive biramous limb with a two-segmented protopodite (fig.1.7A,B and fig. 1.8A). In this scenario, the mandibular gnathal edge is hypothesized to be present on the proximal-most segment of the protopodite, the coxa. I will also present an alternative hypothesis which proposes that the mandibular biting edge is present on a more distal segment of the protopodite (see fig.1.9A).

Serial homology of two structures means that the two structures within the same organism share a common ancestry. If a structure is serially homologous to another structure, then identical genes have been used to pattern both the different structures and it is for this reason that the two structures look identical.

Serial homology requires that the same genes or gene regulatory network has patterned both of the structures giving rise to their morphological similarity. Otherwise, in the unlikely scenario that those similar structures were patterned by different genes in the common ancestor, it would be an example of convergent evolution of those structures within the same organism.

To demonstrate serial homology between the mandible and maxilla protopodite requires showing that the same developmental genes are used to pattern those structures in the ancestral state. To suggest ancestral patterning mechanisms convincingly requires showing that in numerous representative taxa, the genetic mechanisms that are responsible for patterning the mandibular and maxillary protopodite are shared. It also requires an understanding of the evolutionary history of mandible and maxilla diversification from a common, shared, serially homologous biramous limb structure. There is significant diversity of protopodite morphology across arthropod taxa, such as the degree of segmentation of the protopodite and in the numbers of endites associated with each segment. This has implications for attempts to homologize the mandibular protopodite to the protopodites of other appendages.

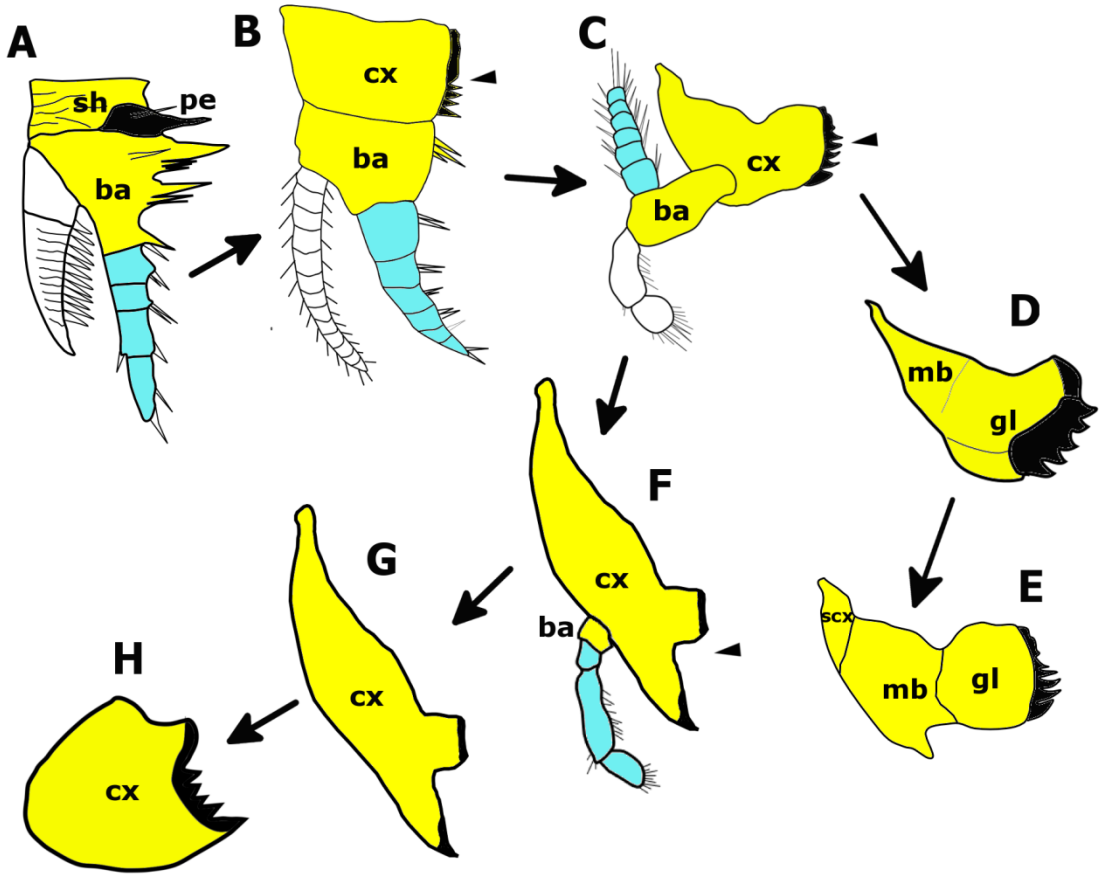

Fig.1.7. Hypothetical mandible evolution from an ancestral biramous limb. The mandibular gnathal edge is located on the proximal protopodite segment (gnathal edge indicated with an arrowhead). The telopodite is highlighted blue, the protopodite is highlighted in yellow, the exopodite is highlighted white. (A) Second post-antennal limb of *Martinssonia elongata.* The limb is biramous, maxilla-like with a developed proximal endite. The unsegmented protopodite is attached to a shaft. (B,C) Hypothetical primitive mandibles derived from the mandible of a Cambrian fossil Maxillopod *Bredocaris* (B) and the biramous copepod mandible (C). The hypothetical plesiomorphic mandible (B,C) evolved from a maxilla-like precuror (A) with reduction of the two palps and reduction of the basis on the telopodite (C). The exopodite palp is lost in numerous crustacean mandibles (F). The telopodite palp is independently lost in numerous pancrustacean mandibles (G). The mandible is structurally modified to possess two articulations and perpendicularly orientated gnathobase in the Dicondylia (H) Myriapod mandibles have lost both palps and the gnathal edge is present on a gnathal lobe (D-E). In this evolutionary framework, the diplopod mandible (E) has evolved an additional protopodite segment. Subcoxa (scx), coxa (cx), basis (ba), shaft (sh), mandible base (mb), proximal endite (pe) and gnathal lobe (gl) are indicated.

Morphological definition of the protopodite

The gnathobasic mandible gnathal edge is a modified endite which is located on the protopodite. Morphologically, the protopodite is defined as the segments at the base of the biramous limb to which the two branches - the telopodite and exopodite - attach. This structure is the biramous limb which is primitive for Arthropoda and present in stem lineage arthropods shown in fig. 1.2D (Boxshall, 2004; Waloszek et al., 2007; Chen, 2009).

Definition of the protopodite on morphological grounds is more difficult for uniramous appendages as there is no exopodite so it is hard to define where the protopodite ends and the telopodite begins. To distinguish the protopodite and telopodite in uniramous limbs requires additional criteria and comparisons to other biramous limbs. The protopodite can also be defined by studying musculature patterns. The protopodite is also characterized by the presence of endites (Snodgrass, 1935; Boxshall, 1998).

The primitive euarthropod protopodite is generally considered to be unsegmented (Boxshall, 2004). In the primitive biramous limb, the telopodite and exopodite are attached to an unsegmented protopodite. This is the case for chelicerates, trilobites and stem lineage euarthropods. Mandibulate arthropods often have up to three segments present on the protopodite, which are called the subcoxa, coxa and basis.

Mandibular protopodite evolution

The mandible has evolved from a primitive biramous limb (fig.1.7). The primitive mandibular palps have clearly been lost independently several times in myriapods (fig.1.7D,E), insects (fig.1.7.G,H) and in certain crustacean taxa. However, the evolution of the mandibular protopodite is less obvious. In the majority of mandibles, the gnathal edge (derived from an endite) is present on the most proximal segment, which is called the coxa (Boxshall, 2004). The only clear exception is the diplopod (millipede) mandible which has a more proximal segment (fig.1.7E).

Boxshall argues that the primitive ancestral mandible protopodite was a two segmented protopodite. The two segments are called the coxa and basis and are present in crustacean mandibles (fig.1.7B,C,F). The crustacean mandibular gnathal edge is present on the more proximal segment, the coxa, whilst the two palps

(exopodite and telopodite) attach to the distal basis segment (see fig.1.7B,C). A coxa and basis were present in the possible stem lineage mandibulate Phosphatocopida (Siveter et al., 2001).

The segmented myriapod mandible

The diplopod mandible protopodite consists of two segments, a proximal cardo and distal stipes (subcoxa and coxa) shown in fig.1.7E and fig.1.8C. The incisor and molar processes are present on the distal segment of the protopodite, the stipes (or coxa), on a movable gnathal lobe. Both the segmented mandible and the movable gnathal lobe of Diplopoda are considered derived structures (Boxshall, 2004; Edgecombe, 2004). Alternatively, the diplopod mandible could represent the primitive state of the mandible if the ancestral mandible had a subcoxal segment as Machida and Kukalova-Peck have hypothesized (Kukalova-Peck, 1998; Machida, 2000; Haas et al., 2001).

There is a resemblance of the movable gnathal lobe of the diplopod mandible (fig. 1.9C) to the proximal endite of putative stem lineage mandibulates such as *Martinssonia* (fig.1.6C). The proximal endite is present on several appendages including the third and fourth post-antennulary segments (mandibular and maxillary) (Muller and Waloszek, 1986; Waloszek et al., 2007).

The diplopod mandible (fig.1.9C) has some similarities to the protopodite of the insect maxilla (fig. 1.9D). Both appendages are monocondylic and have two protopodal segments, a cardo and stipes. The gnathal lobe of the diplopod mandible is connected by a muscle to the stipes. There is a similar muscle connecting one of the maxillary endites, the lacinia, to the stipes that may suggest homology (Snodgrass, 1935; Haas et al., 2001).

Evolution of the Maxilla

In order to homologize the mandible to the maxilla, it is necessary to understand how the maxilla has evolved from the primitive biramous limb from which the mandible has also evolved. However, it is difficult to construct a hypothesis of maxilla evolution from an ancestral biramous limb because of the diversity of maxillary appendages. Despite this it is useful to try to construct a hypothetical scheme in order to highlight the implications of trying to homologize the mandible to the maxilla (see fig. 1.8).

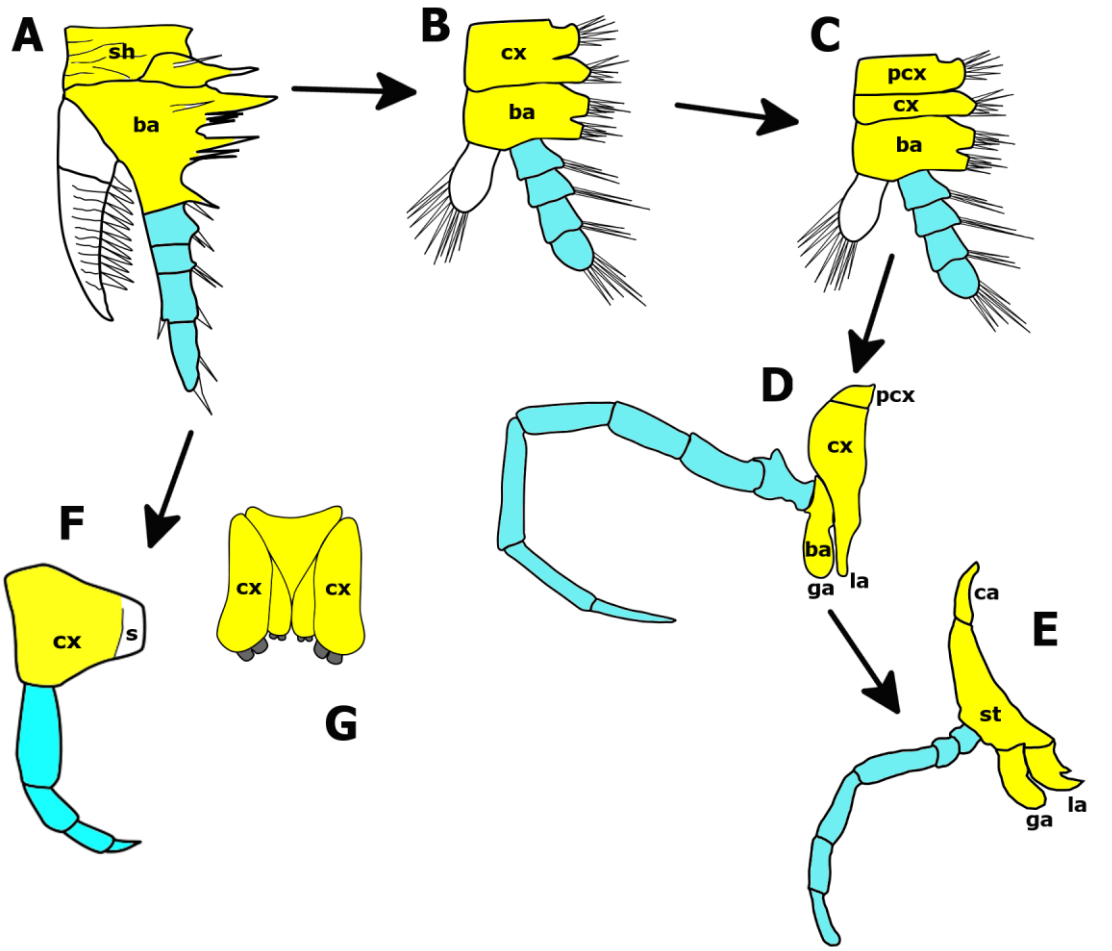

Fig.1.8. Hypothetical maxilla evolution from an ancestral biramous limb. The telopodite is highlighted blue, the protopodite is highlighted in yellow, the exopodite is highlighted white. The precoxa (pcx) or subcoxa (scx), coxa (cx), basis (ba), shaft (sh), proximal endite (pe), cardo (ca), stipes (st), lacinia endite (la), galea endite (ga) are indicated. (A) Third post-antennal limb of *Martinssonia elongata* homologous to the maxilla. The limb is biramous, maxilla-like with several endites, two on the basis, the most proximal endite is more developed. The unsegmented protopodite (ba) is attached to a shaft (sh). (B) Hypothetical ancestral pancrustacean maxilla adapted from Boxshall (Boxshall, 1998). The protopodite has two segments, a coxa and basis with two endites on each present in crustacean taxa such as Cephalocarida, Branchiura and Malacostraca. (C) Crustacean maxilla with three protopodite segments. The coxa of B is divided into a precoxa/subcoxa and coxa present in crustacean taxa such as Copepoda, Ostracoda, Mystacocarida and Remipeda. (D) Maxilla of Archaeognathan hexapod *Pedetontus unimaculatus.* The exopodite has been lost and the protopodite has three segments: precoxa/subcoxa, coxa and basis. The lacinia and galea endites are present on two separate segments unlike the maxilla in E. (E) Typical insect maxilla with a two segmented protopodite with a lacinia and galea endite on the stipes. Boxshall homologises these segments to D as follows: The precoxa/subcoxa is homologous to the cardo. The coxa and basis are fused and are homologous to the stipes. (F) The second chilopod maxilla has lost the endite. The coxa of the maxilla is fused to the sternite (s). both coxae of the maxillae are fused to the sternite to form the coxosternite. The second maxilla is actually homologous to the fourth post-antennal limb of *Martinssonia* which resembles the third post-antennal appendage shown in A. (G) Diplopod gnathochilarium consists of the protopodite of a pair of maxillae fused at the ventral midline that have lost both telopodites.

Maxillules (homologous to the maxilla of both hexapods and myriapods) of crown group crustaceans are diverse in structure. Some crustaceans (like malacostracans) have two segmented maxillules (coxa and basis) with one endite associated with each segment or two endites associated with each segment (see fig.1.8B). Other crustaceans have three segmented maxillules (a precoxa, coxa and basis) with endites on each segment (see fig.1.8C). The basis has one or two endites present. Cambrian arthropods such as *Rehbachiella* and *Martinssonia* also have three or four endites present on the protopodite of the fourth post-antennal appendage (Muller, 1983; Muller and Waloszek, 1986).

Insect maxillae (fig. 1.8E) have two segments, a cardo and stipes, with two endites, a lacinia and galea, attached to the stipes. Archaeognathan hexapods have three segmented protopodites with two endites, the lacinia and galea, present on the two distal-most segments (Kukalova-Peck, 1998; Machida, 2000).

Therefore, one of the most parsimonious reconstructions of maxilla evolution is that the primitive two segmented protopodite evolved into three segments in some crustaceans and primitive hexapods. The two segmented insect maxilla (fig. 1.8E) evolved from a three segmented maxilla present in archaeognathan hexapods (and crustaceans) (fig.1.8D). There are no endites present on the proximal segment of the insect maxilla. This is in contrast to the gnathal edge of the mandible which is present on the proximal segment of the protopodite.

Serial homology of the mandible to the maxilla

The hexapod maxilla (including the insect maxilla) is potentially a useful appendage to study in order to homologize mandibular structures to the primitive biramous limb. The typical hexapod maxilla has more similarities to the primitive biramous limb than the mandible. For example, there is often retention of a palp (the telopodite) and the endites are more similar to those present on biramous limbs.

Machida has hypothesized that the mandibular gnathal edge is homologous to the maxillary lacinia and galea. He based his conclusion on SEM studies on an archaeognathan hexapod *Pedetontus unimaculatus* (Machida, 2000). In the developing embryo, the mandibular appendage has two lobes, an outer and inner lobe. Machida argued that these structures related to the incisor process and molar process and are serially homologous to the galea and lacinia endites of the maxilla respectively. He also

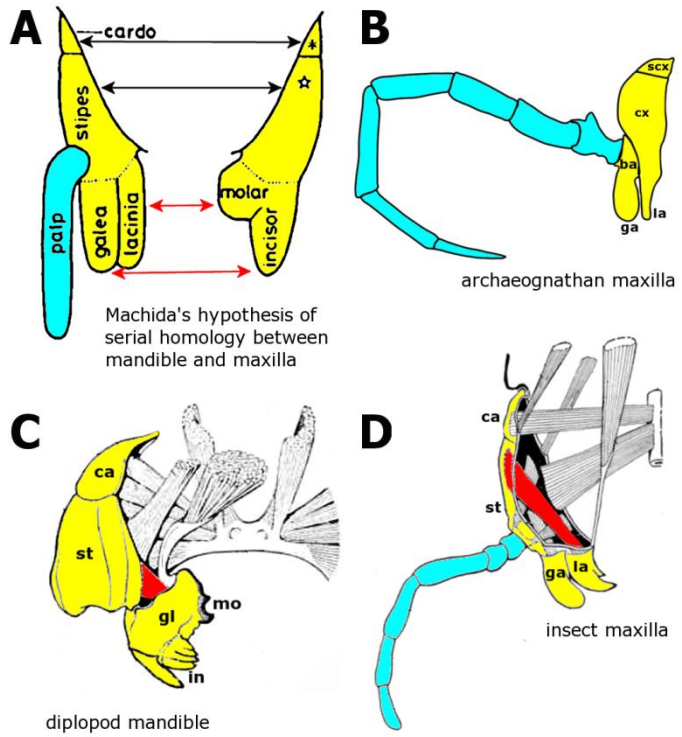

Fig. 1.9. Serial homology of the mandible to the maxilla. Figures A,B are adapted from Machida (2000). Figures C,D are adapted from Snodgrass (1935). (A) Machida's hypotheses of homology between the mandible to the maxilla. The subcoxa (asterisk) and coxa (star) of the mandible are proposed to be homologous to the cardo and stipes of the maxilla (black arrows). The molar (inner lobe) is homologous to the lacinia. The incisor process (outer lobe) is homologous to the galea (red arrows)(Machida, 2000). The two endites, the molar and incisor processes, are present on the more distal coxal segment (star) which is homologous to the stipes. (B) Maxilla of archaeognathan hexapod *Pedetontus unimaculatus*. The protopodite is divided into three segments: subcoxa, coxa and basis. The lacinia and galea are present on separated segments, unlike the mandible incisor and molar processes shown in A. If this maxilla is primitive, it means that the insect maxilla stipes (D) is likely to consist of two segments, coxa and basis, that have fused. (C) Diplopod mandible is divided into two segments, a cardo and stipes. A muscle attaching the gnathal lobe to the stipes is highlighted in red. (D) Generalized insect maxilla. Muscle attaching the lacinia to the stipes is highlighted in red, which could be homologous to the highlighted muscle shown in C. The telopodite is highlighted blue, the protopodite is highlighted in yellow, the exopodite is highlighted white. The basis (ba), incisor process (in), molar process (mo), cardo (ca), stipes (st), lacinia endite (la), galea endite (ga) are indicated.

argued that the mandible consisted of two segments (a subcoxa and coxa) that are serially homologous to the cardo and stipes of the maxilla. Machida therefore interprets the mandible gnathal edge as consisting of two endites, and that the primitive mandible had a subcoxal segment (see fig.1.9A).

Contra Machida

Machida's hypothesis is a little simplistic. Evidence for his hypothesis is provided from comparison of the embryonic development of the mandible and maxilla from one species and does not take into account the diversity of maxillary appendage structures that are found in arthropods. Nor does he take into account the evolutionary history of the maxilla. Relating the maxilla to the mandible in terms of serial homology requires understanding the evolution of these two appendages from when they diverged from an identical serially homologous biramous limb over 500 million years ago (compare fig.1.7 to fig. 1.8).

Boxshall disagrees with two particular aspects of Machida's hypothesis. Machida homologizes the maxillary lacinia and galea to the mandibular molar and incisor processes. However, the archaeognathan maxilla has the lacinia and galea present on separate segments, a coxa and basis (see fig.1.9B) which suggests that the insect maxilla has evolved by fusion of the coxa and basis to form the stipes. This fusion resulted in the two endites being present on the same segment in the insect maxilla. However, there is no arthropod mandible that has the incisor and molar processes present on separate segments and so Machida's hypothesis is considered unlikely.

Also, Machida hypothesizes that the mandible has a more proximal subcoxal segment and that the gnathal edge is present on a distal segment of the protopodite (fig.1.9A). However, in the majority of arthropods, the mandible gnathal edge is present on the proximal segment of the protopodite (see fig.1.7B-D,F-H) and not a distal segment as Machida hypothesized (Boxshall, 2004).

The subcoxa origin of the pleuron.

Hexapod legs have one obvious protopodite segment, the coxa. It is hypothesized that hexapods also have a subcoxa, an additional appendage segment, which is incorporated into the body wall to from the pleuron. This is the known as the subcoxal theory of the pleuron (Snodgrass, 1935; Boxshall, 2004). Pleural sclerites are also present in myriapods (Bäcker et al., 2008). The pleuron is likely to be an example of convergent evolution between Hexapoda and Myriapoda, to terrestialization by providing additional support to the basal joint of the walking legs. In numerous

crustacean trunk limbs with a three segmented protopodite, the proximal segment, the precoxa, is incorporated into the body wall.

There is potential to homologize parts of proximal leg segments to the proximal protopodite segments of the gnathal appendages. If the pleural sclerites derive from a subcoxal segment (the subcoxal hypothesis), then there could be a homologous relationship between the subcoxa of the legs and the proximal protopodite segments of other appendages (Boxshall, 2004). In which case, the subcoxa could be homologous to the proximal segments of the gnathal appendages.

Hypotheses of embryonic mandible structure

With consideration of the above, there were three specific hypotheses that I wanted to test to examine the serial homology of the mandible to other appendage types. Firstly I wanted to determine if the mandible gnathal edge derived from two endites as Machida has suggested, or whether it is derived from one endite. If it was shown that the mandible is derived from one endite, this would contradict Machida's hypothesis of serial homology between the mandible and maxillary endites. Secondly I wanted to determine if there was any molecular evidence for the division of the insect mandible into a subcoxa and coxa. Evidence of a more proximal mandibular subcoxa could support serial homology of the mandibular subcoxa to the maxillary cardo as Machida has hypothesized. And finally, I wanted to provide molecular evidence for the subcoxal origin of the pleuron which could provide evidence for the serial homology of the leg subcoxa to the gnathal appendages.

The question of appendage segment homology of the gnathal appendages is a difficult problem to resolve with comparative morphology alone. The problem is exacerbated by the lack of certainty regarding the higher level phylogeny of some arthropod groups, and the difficulty in reconstructing the primitive protopodite from the diversity of arthropod appendage forms. By studying genes that are involved in patterning the protopodite, it might become easier to determine serial homology of different segments and endites, and homology of protopodite segments between different arthropod taxa.

1.7 Molecular development of the mandible

If the mandible gnathal edge, and the mandible appendage itself, is a homologous character across Mandibulata, it would suggest that there may be significant similarities between the mandible developmental pathway of diverse taxa of Mandibulata. Hox genes have a conserved role in patterning segments of arthropods. In addition to Hox genes, research in *Drosophila*, a non-mandibulate arthropod, has shown that another gene *cap'n'collar (cnc)* functions to modify Hox gene function and is required to pattern the mandibular segment.

The appendages of arthropods have evolved and diversified since the last common ancestor, which possessed identical serially homologous biramous limbs. Arthropod appendages are present in pairs on individual segments. The segments on which the anterior appendages are found have maintained their identity across arthropod lineages. The evidence for this is from several sources, one of the most powerful is the conserved segmental expression (especially the anterior limit of expression) of a class of genes known as Hox genes.

There are several additional genes that have a conserved role in patterning the arthropod limb which can be used to study the developing mandibular appendage of *Tribolium*. These are the genes that pattern the proximal-distal axis of the limb (Angelini and Kaufman, 2005) and the notch signalling pathway which has a role in segmenting the limb (Rauskolb, 2001; Prpic and Damen, 2009). As these genes have a conserved role in patterning the arthropod limb, they can be used to study the homology of different limb segments and endites. The relative position of the PD domain genes to the notch signalling pathway, which demarcates appendage segments, may reveal the precise identity of particular segments as they have evolved throughout the Arthropoda and determine whether there are any similarities that are suggestive of serial homology.

PD domain genes

A common mechanism of gene regulation in embryonic development is the division of regions along an axis by genes that are expressed in broadly overlapping domains that activate or repress downstream genes according to their position along the axis. Such a situation occurs in along the *Drosophila* anterior/posterior (A/P) axis as

mediated by both Gap genes and Hox genes but a similar mechanism patterns the Proximo-Distal (PD) axis of the appendages.

Several genes in *Drosophila* have been discovered that define the PD axis of leg appendages. Four of the most important genes are the transcription factors *Distal-less (Dll)*, *dachshund (dac)*, *homothorax (hth)* and *Extradenticle (Exd)*. These genes and the role they perform in patterning the proximal-distal axis of the limb has been well studied in the leg imaginal discs of *Drosophila* (Panganiban et al., 1994; de Celis et al., 1998; Wu and Cohen, 1999; Dong et al., 2001; Rauskolb, 2001; Schram and Koenemann, 2001; Hao et al., 2003; Kojima, 2004).

These four PD domain genes, *Dll, dac, hth* and *Exd,* set up five overlapping domains within the PD axis which define particular regions of the limb: 1) *hth, 2) hth + dac+ dll, 3) dac, 4) dac + dll, 5) dll. Exd* and *hth* are cofactors that pattern the base of an appendage (Jaw et al., 2000; Prpic and Telford, 2008). *Dac* patterns the medial portion of an appendage whilst *Dll* is responsible for both limb outgrowth and patterning the distal tip of an appendage (Rauskolb, 2001; Inoue et al., 2002).

Dll and *dac* function in a manner analogous to Gap genes, embryos that are mutant for either of these two genes have deletions of tissue that relate to their expression domain, there is no transformation of tissue type. *Dll* is expressed distally in the tibia, tarsi and pretarsi and in a proximal domain between the trochanter and femur (Gonzalez-Crespo and Morata, 1996). *dac* is expressed in a medial intermediate domain in the imaginal disc (Mardon et al., 1994; Rauskolb, 2001).

Consistent with these gene expression patterns, hypomorphic *Dll* mutant flies lack the tibia, tarsi and pretarsi segments (Cohen and Jurgens, 1989; Panganiban, 2000). While flies that are mutant for *Dac* have the femur, tibia and proximal three tarsus segments fused and condensed but not segments proximal or distal to its domain are unaffected (Mardon et al., 1994).

Two genes that pattern the base of the legs are *homothorax* and *Extradenticle*. Exd and Hth act as a gene pair. Exd requires Hth as a cofactor for nuclear localization in order to become active. Nuclear Exd is referred to as nExd. *Hth* and *Exd* do not function like gap genes but rather mutants of these two cofactors results in a failure of the leg segments to form (Abu-Shaar and Mann, 1998; Jaw et al., 2000; Casares and Mann, 2001; Prpic and Telford, 2008).

Comparison of the PD domain genes across Arthropoda

Comparisons of gene expression patterns and of gene function have shown the expression of the PD domain genes to be conserved in leg appendages across Arthropoda in all studied organisms (see fig. 1.10). Homologues of *Dll* are expressed in the distal parts of all appendages (except the mandible) (Panganiban et al., 1997), *dac* is expressed in the medial portion. *hth* and *Exd* co-expression, which is necessary for their function, is conserved in the proximal part of arthropod appendages (Jaw et al., 2000; Prpic et al., 2003; Angelini and Kaufman, 2005; Prpic and Telford, 2008).

Species that have been studied include the insects: *Tribolium castaneum* (Beermann et al., 2001; Prpic et al., 2001; Prpic et al., 2003), *Oncopeltus fasciatus* (Angelini and Kaufman, 2004), *Gryllus bimaculatus* (Inoue et al., 2002), *Acheta domesticus* (Abzhanov and Kaufman, 2000b) and *Schistocerca americana* (Giorgianni and Patel, 2004; Jockusch et al., 2004). Crustaceans that have been studied include *Porcellio scaber* (Abzhanov and Kaufman, 2000b) and *Parhyale hawaiensis* (Prpic and Telford, 2008; Liubicich et al., 2009). There has only been analysis of one myriapod, the Diplopod *Glomeris marginata (Prpic and Tautz, 2003).* Several Chelicerates have been studied and include *Steatoda triangulosa* (Abzhanov and Kaufman, 2000b)*, Cupiennius salei* (Prpic and Damen, 2004), and *Acanthoscurria geniculata* (Pechmann and Prpic, 2009).[7]

Interestingly, PD domain genes have been studied in an onychophoran *Euperipatoides kanangrensis* that possesses non-segmented lobopodial limbs. The PD domain genes are expressed in a similar proximal-distal order to those of arthropods indicating that the PD axis specifying function of the PD domain genes probably

[7] Expression of *Dll* has been investigated in significantly more diverse taxa in addition to those mentioned above. *Dll* expression has been investigated in several insects: *Manduca* Tanaka, K. and Truman, J. W. (2007) 'Molecular patterning mechanism underlying metamorphosis of the thoracic leg in Manduca sexta', *Dev Biol* 305(2): 539-50., *Precis, Athalia, Thermobia, Lepisma, Folsomia, Xenylla.* In several crustaceans *Artemia, Mysidopsis, Daphnia, Nebalia, Triops, Sacculina, Thamnocephalus.* And also in the chelicerates *Achaearanea,* Arachae *Argiope,* Xiphosuran *Limulus* and an onychophoran *Peripatopsis* Angelini, D. R. and Kaufman, T. C. (2005) 'Insect appendages and comparative ontogenetics', *Dev Biol* 286(1): 57-77.

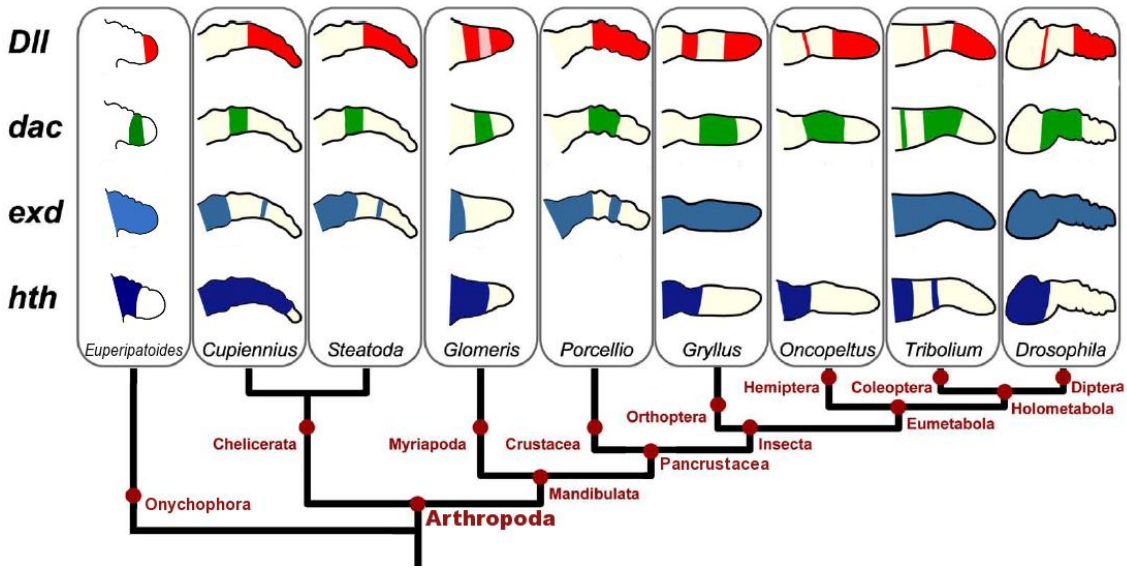

Fig.1.10. Conservation of PD domain gene expression across Panarthropoda. Figure is adapted from Angelini and Kaufman (2005). Expression of the PD domain genes *Dll, dac, hth* and *exd* is shown schematically for numerous arthropods and an onychophoran. *Dll* is expressed in the distal part of each limb. *dac* is expressed in the medial region. There is more variation of *hth* and *exd* expression, however, co-expression of *hth* and *Exd* necessary for function is conserved at the base of the limb. References to the expression patterns of these genes in different taxa are indicated in the text.

predates the formation of jointed arthropod appendages (Panganiban et al., 1997; Janssen et al., 2010).

Wherever examined, the function of the PD domain genes has been shown to be conserved. The homologue of *Dll* was investigated in *Tribolium* where there is truncation of leg segments distal of femur in *Tc Dll* mutants (Beermann et al., 2001). *Dll* knock down by RNAi in *Oncopeltus* resulted in deletion of segments distal of femur (Angelini and Kaufman, 2004). In *Daphnia magna,* RNAi resulted in truncation of the second antenna (Kato et al., 2011).

Outside of mandibulates, *Dll* function has been examined in the spider *Cupiennius* (Schoppmeier and Damen, 2001) and *Achaearanea* (Prpic, personal communication) and the two-spotted spider mite *Tetranychus urticae* (Khila and Grbic, 2007) where distal appendage regions were missing when the gene's function was disrupted. *dac* function has been investigated in *Oncopeltus* and knockdowns affected the medial region of limbs (Angelini and Kaufman, 2004). *hth/Exd* function has been investigated in the cricket *Gryllus* (Mito et al., 2008; Ronco et al., 2008) and the milkweed bug *Oncopeltus* (Angelini and Kaufman, 2004).

The protopodite-telopodite boundary from the molecular perspective

The protopodite is defined morphologically as the proximal part of the limb to which the telopodite and endopodite attach. It is of interest as to whether the PD domain genes are seen to define the protopodite-telopodite boundary from a molecular perspective, and whether this would agree with morphological analyses.

Gonzalez-Crespo and Morata hypothesised that *exd* expression related specifically to the protopodite and *dll* expression to the telopodite (Gonzalez-Crespo and Morata, 1996). Whilst the broad outline of this is true, at different stages of leg development during embryogenesis, the PD domain genes can have different regulatory interactions (Rauskolb, 2001). For example, early in limb development *Dll* is expressed in cells that will form the protopodite (McKay et al., 2009).

Initial research into the division of the protopodite and telopodite looked at the expression of genes as markers for these fundamental leg divisions. *Dll* and *Dac* expression domains were used as markers for the telopodite (Gonzalez-Crespo and Morata, 1996; Estella and Mann, 2008; Estella et al., 2008). There are no obvious markers for the protopodite. nExd is expressed in both the coxa and trochanter. The trochanter is not considered to be part of the protopodite[8] and therefore nullifies nExd's use as a protopodite marker (McKay et al., 2009). In addition, during the earliest stage of leg primordia formation, Hth-nExd are co-expressed with *Dll.* Later in leg development, Hth-nExd and *Dll* are mostly mutually exclusive. One region that does co-express Hth-nExd and *Dll* gives rise to the trochanter (Abu-Shaar and Mann, 1998; Rauskolb, 2001).

Dll enhancers and the protopodite-telopodite boundary

Proximal and distal cell populations that broadly relate to the protopodite and telopodite in the developing limb have the ability to sort themselves from one another based on positional signalling cues. It has been shown that both *hth* and *Dll* contribute to this process. Mitotic cell clones that express *hth* ectopically in the leg can repress *Dll* and *dac*. These *hth* expressing clonal cells migrate towards the protopodite and body wall. In mitotic protopodite cell clones that have lost *hth,* the proximal leg segments

[8] Although the trochanter is considered part of the protopodite by Kukalova-Peck. See Haas, M. S., Brown, S. J. and Beeman, R. W. (2001) 'Homeotic evidence for the appendicular origin of the labrum in *Tribolium* castaneum', *Dev Genes Evol* 211(2): 96-102.

fuse. Cell that ectopically express *Dll* in the proximal part of the limb primordia are excluded from this part of the limb, while cell clones that have lost *Dll* function cannot remain in the developing leg and migrate out of the leg appendage primordia (Wu and Cohen, 1999; Angelini and Kaufman, 2005; McKay et al., 2009).

Dll is important for both the initial specification of leg primordia (which includes the protopodite) and for differentiating the telopodite from the protopodite in *Drosophila*. McKay *et al.* (2009) determined a high resolution cell fate map of the *Drosophila* leg primordia. The authors showed that the activity of *Dll* is dependent on several different *Dll* enhancers at different stages of leg development in the fly embro and is responsible for both telopodite and protopodite progenitors (McKay et al., 2009).

One of these enhancers is *Dll304.* Cells that have *Dll* expression activated by the *Dll304* enhancer are multipotent and will give rise to both protopodite and telopodite cell populations. *Dll304* is activated by *wg* (providing anterior-posterior positional information) and restricted by *Dpp* dorsally and by *EGFR* signalling ventrally. Another enhancer, the *LT* enhancer (leg trigger enhancer) is activated in cells that give rise to the telopodite, and is activated by both Wg and Dpp (Estella et al., 2008; McKay et al., 2009).

McKay *et al.* (2009) concluded that *Dll304* is responsible for all leg cell populations including the protopodite. Lineage restriction of cell populations between the protopodite and telopodite occurs when the *LT* enhancer is activated. Cells that express *Dll* via the *LT* enhancer become the telopodite. Cells that had activated *Dll304* but not the *LT* enhancer assume protopodite identity.

The trochanter presents an unusual situation. As noted above, the trochanter is marked by both *Dll* expression and hth-nExd expression late in leg formation. Cells from both the protopodite and the telopodite can contribute to the trochanter. Therefore the authors note that the protopodite-telopodite division does not constitute a classical definition of a compartment boundary as there is a region, the trochanter, that can incorporate both protopodite and telopodite cell lineages (McKay et al., 2009).

It would be of considerable interest to determine whether a similar division of protopodite and telopodite cell populations occurs in other arthropod taxa. Currently, there are no species that can be practically studied in such detail outside of *Drosophila.*

But the potential is there to uncover a conserved molecular protopodite-telopodite division mechanism. An understanding of such mechanisms would greatly aid our attempts to homologize limb segments between different limbs and different arthropod taxa, particularly if the protopodite/telopodite division mechanism was studied in a biramous appendage. This would unambiguously relate the protopodite-telopodite cell lineages to the morphological definition of the protopodite and telopodite.

The use of *Dll* expression as a telopodite marker and the resolution of the gnathobasic or telognathic mandible controversy

Studies have used *Dll* as a marker for the telopodite and have been able to resolve a controversy concerning the gnathobasic or telognathic nature of the mandible in different arthropod groups. The question concerned the mandibular biting edge and whether it had evolved from either the protopodite or the telopodite of the ancestral appendage. Manton hypothesized that the mandible of hexapods and myriapods had evolved from the tip of the ancestral limb, the telopodite, and was therefore telognathic. Whereas the mandible of the crustaceans was less controversially considered gnathobasic, as in numerous representatives of crustaceans the biting edge is present on the base of the mandibular apppendage with the telopodite attached to the protopodite as a palp (Manton, 1964; Manton, 1977).

Manton's interpretation has been shown to be incorrect through the study of *Dll* expression which is expressed in the distal tips of developing appendages (Panganiban et al., 1994; Panganiban et al., 1997), but not the developing mandibular appendage (Niwa et al., 1997; Popadic et al., 1998; Scholtz et al., 1998). *Dll* expression is typically lacking in the mandibular segment of myriapods and hexapods which demonstrates that the functional biting edge of the mandible has a protopodal, and therefore gnathobasic origin, confirming Snodgrass' gnathobasic interpretation of the insect and myriapod mandible (Snodgrass, 1938; Snodgrass, 1950). The lack of a palp in insects and myriapods is therefore interpreted as a loss of the telopodite, which is still present in numerous crustacean species. In these species, *Dll* is expressed in the developing palp of mandibles with palps, for example the amphipod *Gammarus pulex* and the mysid *Mysidium columbiae* (Browne and Patel, 2000).

1.8 Homology of anterior arthropod segments

Hox genes

One class of genes that are important for patterning segments in arthropods, and other segmented animals are the Hox genes. Hox genes play a central role in patterning segments and giving them their identity. Hox genes are master regulatory genes that activate downstream targets that pattern segments (Carroll et al., 2004).

Hox genes are segment patterning genes that are expressed in a co-linear manner along the anterior-posterior axis of diverse bilaterians. Hox genes are present in clusters, with varying degrees of organisation from highly compact non-interrupted clusters with genes present in the same orientation as present in vertebrates, less compact but still ordered clusters like in *Tribolium* (Brown et al., 2002a), to split clusters with genes present in different orientations as in *Drosophila* (Duboule, 2007).

The gene order (3' to 5') of the Hox genes in organised Hox gene clusters mirrors the order of expression from anterior to posterior segments. Posterior Hox genes have dominance over anterior Hox genes when co-expressed in the same segment.

Hox gene null mutants often have one or more segments changing their identity to that of another segment, a transformation known as a homeotic transformation. Homeotic transformations often result in the segment taking the identity of the adjacent anterior segment (Veraksa et al., 2000). In *Tribolium,* if a segment is lacking Hox gene expression altogether, one result that occurs is transformation of post-antennal appendages into antennal identity (Brown et al., 2002b).

Studies investigating the expression of Hox genes in Arthropods have revealed considerable similarities in anterior expression boundaries. These anterior boundaries have been used to homologize segments between taxa (Hughes and Kaufman, 2002a). For example, the anterior expression boundary of the hox gene *Deformed (Dfd)* is expressed in the mandibular segment in all studied Mandibulates. The anterior boundary of *Dfd* in Chelicerates is expressed in the first leg segment. Considering the correspondence of the anterior boundaries of other Hox genes across different groups (see fig.1.11), the mandibular segment of mandibulates is homologous to the first leg

segment of Chelicerates (Damen et al., 1998; Telford and Thomas, 1998a; Hughes and Kaufman, 2002a).

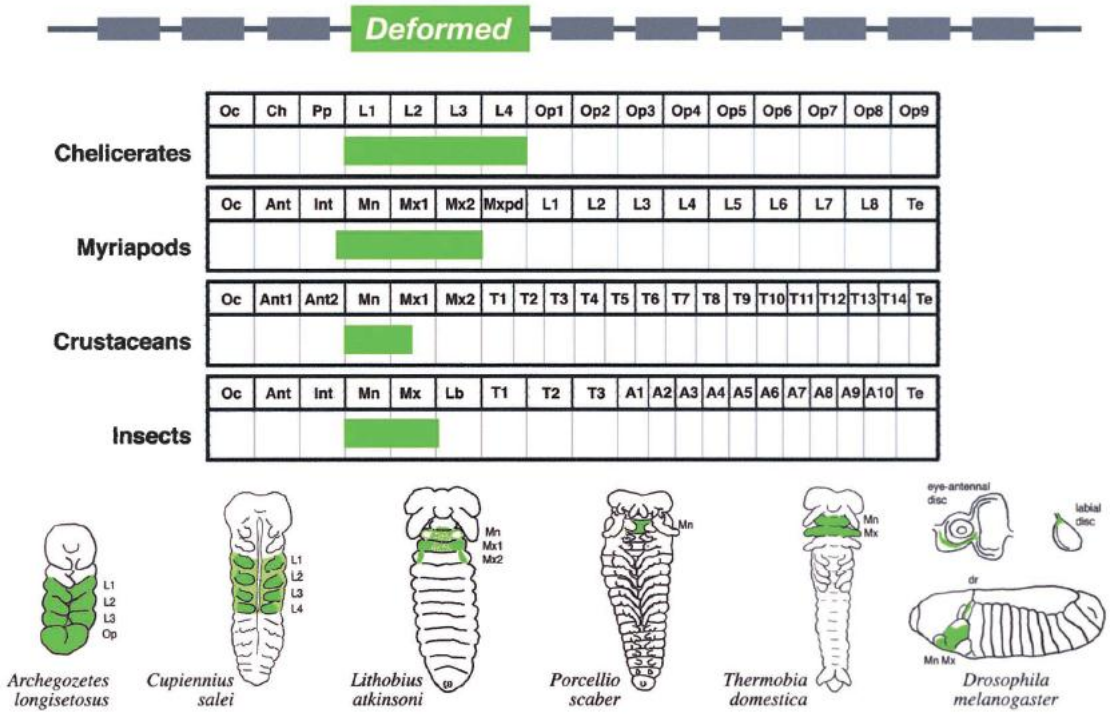

Fig.1.11. Expression of *Deformed* homologizes the mandibular segment to the first leg segment of Chelicerata. Figure is adapted from Hughes and Kaufman (2002a). The segmental abbreviations are as follows: ocular (Oc), chelicerae (Ch), pedipalps (Ped) and four Leg segments (L1-L4) opisthosomal segments (Op), antennal (ant), intercalary (Int), mandibular (Mn), first maxilla (Mx1), second maxilla (Mx2), maxilliped (Mxpd), labial (Lb), thoracic (T), abdominal (A), Telson (T).

Deformed (Dfd) expression is conserved in the mandibular and maxillary segments across mandibulates. Expression in centipedes also includes the 2nd maxillary segment. Where *Dfd* function has been investigated, it is responsible for patterning the mandibular and maxillary segments (Mahaffey et al., 1989; Diederich et al., 1991; Abzhanov and Kaufman, 1999a; Brown et al., 2000; Hughes and Kaufman, 2002a; Hughes and Kaufman, 2002b; Rogers et al., 2002; Janssen and Damen, 2006).

There are other Hox genes as well as *Dfd* that are expressed in the mandibular segment of some mandibulates, such as Hox3 and *pb* (Shippy et al., 2000b; Hughes and Kaufman, 2002a; Hughes et al., 2004). Although these two genes are typically expressed in the mesoderm of these appendages and therefore are not responsible for patterning the appendage, as appendages are primarily patterned from the ectoderm. However, to date there has been no discovery of a Hox gene that is responsible for the specific identity of the mandibular segment.

cap'n'collar differentiates the mandibular segment from the maxillary segment

One gene that differentiates the mandibular segment from the maxillary segment, at least in *Drosophila,* is *cap'n'collar* (*cnc*). *cnc* is a basic Leucine zipper family gene (bZIP), members of which are found in all organisms and Orthologues of *cnc* are found throughout Bilateria.

If the mandible is a synapomorphy of Mandibulata, then genes such as *cnc* that specify the mandibular segment and differentiate the mandible from other gnathal appendages may be conserved in diverse mandibulate taxa, and not have such a role outside of mandibulates such as chelicerates.

The view from *Drosophila* genetics

An investigation into the genetics of the fruit fly is a prerequisite in any venture into the genetics of any arthropod. Much work has been done following the work done in *Drosophila* by comparing classes of developmental genes to other organisms, such as maternal genes, Gap genes, Head gap genes, Pair-rule genes, segment polarity genes, Hox genes and PD domain genes. It was therefore to *Drosophila* that I turned to in order to chose candidate mandibular patterning genes.

The *Drosophila* gnathocephalon and pseudocephalon

In a number of morphological respects, *Drosophila* larvae and adults like other cyclorraphous dipterans are derived (Grimaldi and Engel, 2005). *Drosophila* larvae are without a head (*acephalic*) and undergo a derived mode of development known as head involution which involves the anterior ectoderm invaginating and moving to the interior (Jurgens et al., 1986; Finkelstein and Perrimon, 1991; Rogers and Kaufman, 1997; Schinko et al., 2008). Adult *Drosophila* have lost the gnathal appendages of the mandible, and the maxilla is highly reduced. The feeding appendage is a proboscis derived almost wholly from the labial appendage (Merrill et al., 1987; Chadwick et al., 1990; Abzhanov et al., 2001).

Drosophila larvae do not have gnathal appendages (compare fig.1.12A with fig.1.12B) but do possess gnathal lobes, structures from which appendages are formed in other insects (compare fig.1.12C with fig. 1.12D). The mandibular, maxillary and labial gnathal lobes which form the gnathocephalon, develop into the pseudocephalon

at the anterior of the embryo (see fig.1.12A). The pseudocephalon is surrounded by the cuticle from the first thoracic segment (Regulski et al., 1987).

While the gnathocephalon is homologous to the gnathocephalon of other developing mandibulate embryos, the pseudocephalon is highly derived and bears almost no resemblance to other mandibulate larval heads. There are, however, larval structures derived from the mandibular and maxillary segments which have been subject to genetic analysis and laser ablation studies to determine their segmental origin (Jurgens et al., 1986) (see fig.1.12A,C).

The axis of the flattened gnathal lobes has traditionally been described as ventral-lateral to dorsal in *Drosophila* genetics. The ventral-lateral to dorsal axis is the same as the proximal to distal axis described in other mandibulate arthropods. Proximal refers to the base and distal refers to the tip of the developing appendage[9]. For ease of comparison, the nomenclature used to describe the gnathal lobe axis as proximal to distal will be used throughout this thesis in preference to the nomenclature used to study *Drosophila*.

The structures which are derived from these proximal and distal domains in *Drosophila* are homologous to structures found in other mandibulate hexapods. The appendage-less larvae of *Drosophila* still possess sense organs and cirri that are the only remaining structures left from the loss of the gnathal appendages present in the majority of insects (Jurgens et al., 1986; Panganiban, 2000). The palp of mandibulate hexapods is homologous to the distal domain of the maxillary lobe of *Drosophila* and the endites are homologous to the proximal domain of the maxillary lobe. The sense organs that develop from the gnathal lobes of *Drosophila* embryos are homologous to those found on insect appendages (Behan and Ryan, 1978; Jurgens et al., 1986). The maxilla sense organ is homologous to the sense organ found on the maxilla palp. The cirri and ventral organ are homologous to structures on the maxilla endites (Jurgens et al., 1986).

[9] To further clarify: The *Drosophila* maxillary lobe consists of ventral-lateral and dorsal domains which are homologous to the distal and proximal domains of mandibulate Hexapods. The dorsal domain of the *Drosophila* maxillary lobe is homologous to the *Tribolium* maxilla palp, whilst the *Drosophila* ventral-lateral domain is homologous to the *Tribolium* maxillary endites.

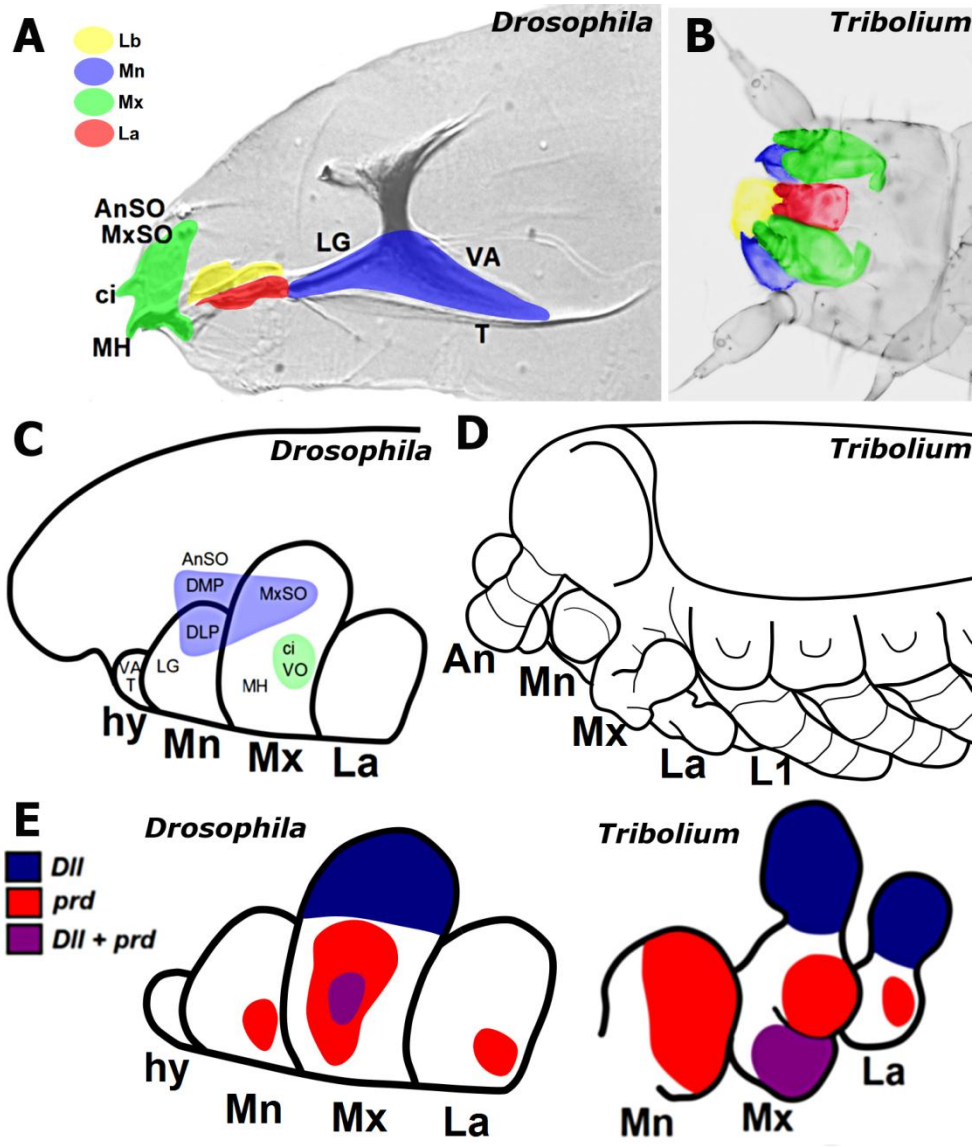

Fig.1.12. Comparison of mandibulate (represented by *Tribolium)* and *Drosophila* larval and embryonic morphology. (A-C) adapted from Jurgens *et al.* (1986). Labrum (Lb), antennal (An) mandibular segment (Mn), maxillary segment (Mx), labial segment (La), first leg (L1) segments are indicated. (A) *Drosophila* larva head with segment origins indicated by colour. Mouth hooks (MH), cirri (ci) and the ventral organ (VO) are derived from the maxillary segment. Lateralgraten (LG) of the hypopharyngeal skeleton are derived from the mandibular segment. The ventral arms (VA) and T-ribs (T) are derived from the hypopharyngeal lobes (Jurgens et al., 1986; Finkelstein and Perrimon, 1991; Rogers and Kaufman, 1997). The antennal sense organ (AnSO) is derived from the antennal segment. Most of the maxillary sense organ is derived from the distal portion of the maxillary gnathal lobe. (Jurgens et al., 1986). (B) *Tribolium* larval head with gnathal appendages highlighted. (C) Schematic of the *Drosophila* embryo with segmental origins of larval structures indicated. The hypopharyngeal lobes have been shown to derive from the mandibular segment (Economou and Telford, 2009). The maxillary sense organ (MxSO) has diverse segmental origins (shaded in blue). The dorso-medial papilla (DMP) and the dorso-lateral papilla (DLP) are derived from the antennal and mandibular segments respectively. The ventral organ and cirri, are derived from the proximal part of the maxillary lobe (shaded in green) (D) *Tribolium* embryo schematic showing the developing segmental appendages. (E-F) Expression of *Dll* (blue) and *prd* (red) in the gnathocephalon of *Drosophila* (E) and *Tribolium* (F). The distal part of the maxillary gnathal lobe is marked by a distal domain of *Dll* expression. The proximal part of the maxillary gnathal lobe is marked by *prd* expression. In *Drosophila,* the ventral organ and cirri, are derived from the proximal part of the maxillary lobe (shaded in green in C) and are homologous to the *Tribolium* endites.

Expression patterns of two genes, *Dll* and *prd* are expressed in proximal domains in the maxillary gnathal lobe similar to that of mandibulate insects. The proximal expression domains of *Dll* and *prd* are homologous to the proximal expression domains found in the endites of *Tribolium*. *Dll* is also expressed in a distal domain in *Drosophila* that is likely to be homologous to the distal domain of *Dll* expression in *Tribolium* and other insects. These expression patterns are consistent with the homology of the proximal domain of the *Drosophila* maxillary lobe to the maxillary endites of *Tribolium* and other mandibulate arthropods (see fig. 1.12E,F).

1.9 Mandibular segment patterning genes in *Drosophila*.

Deformed

Dfd is expressed in the mandible and maxillary segments of all studied mandibulates. The most thorough genetic analysis of *Dfd* in an insect, crustacean or myriapod has been in *Drosophila. Drosophila* larvae lack appendages but there are some structures derived from the mandibular and maxillary segments which can be used as markers during the larval stage to determine the segmental patterning function of *Dfd* (see fig 1.12A,C).

Drosophila that are mutant for *Dfd* are missing maxillary and mandibular derived structures. In particular, *Dfd* null mutant flies are missing the cirri, ventral organ, mouth hooks which are of maxillary segment origin and the Lateralgraten and the dorso-lateral papilla of the maxillary sense organ which are of mandibular segment origin (Merrill et al., 1987; Regulski et al., 1987; McGinnis et al., 1998; Brown et al., 1999; Veraksa et al., 2000). Ectopic expression of *Dfd* results in ectopic maxillary structures such as cirri, ventral organs and mouth hooks on the labial and thoracic segments (O'Hara et al., 1993). Several target genes have been shown to be regulated by *Dfd* in the developing embryo.

cap'n'collar

There is no Hox gene that differentiates the mandibular segment from the maxillary segment in *Drosophila*. Experiments have shown, however, that *cnc* differentiates the mandibular segment from the maxillary segment. *cnc* is necessary for the development of mandibular derived structures and achieves this at least in part by repressing the activity of *Dfd* in the mandibular segment. For its role in modifying

Dfd function, it has been labelled a Hox modulator gene (Mohler et al., 1995; McGinnis et al., 1998; Veraksa et al., 2000).

cnc null mutants lose labrum and mandibular segment derived structures and have a duplication of maxilla derived structures. Specifically the duplicated maxillary structures include the mouth hooks of the hypopharyngeal skeleton, cirri and the ventral organ. Mandibular segment derived structures are lost such as the Lateralgraten. Hypopharyngeal lobe derived structures, the ventral arms and T-ribs of the hypopharyngeal skeleton, which are themselves derived from the mandibular segment (Economou and Telford, 2009), are also lost in *cnc* null mutants (Mohler et al., 1995; McGinnis et al., 1998).

Whilst data from *Drosophila* are useful for finding candidate genes for mandibular segment patterning, there are limits as to what can be understood from *Drosophila. Drosophila* does not possess a mandibular appendage in either the larval or adult form. In addition, the *Drosophila* leg protopodite is lacking endites. Given that the mandibular appendage is a protopodite with a pronounced modified endite, *Drosophila* is limited as to how much it could inform us about the development of the mandible appendage.

The majority of research into the function of genes patterning arthropod gnathal appendages has focused on insects that possess derived mouthparts. In terms of the evolution of the mandible these studies have discussed mandibular evolution in terms of evolution from a typical mandibular appendage (the ancestral mandible and similarly structured mandibles) into highly derived structures such as the proboscis of adults of the dipteran *Drosophila melanogaster* (Chadwick et al., 1990; Abzhanov et al., 2001; Joulia et al., 2005; Joulia et al., 2006), the development of the gnathal lobes and pseudocephalon in embryos and larvae of *Drosophila* (Regulski et al., 1987; Mohler, 1993; Mohler et al., 1995; McGinnis et al., 1998; Veraksa et al., 2000), and the stylet of the hemipteran *Oncopeltus fasciatus* (Hughes and Kaufman, 2000; Angelini and Kaufman, 2004; Angelini et al., 2005).

1.10 The red flour beetle *Tribolium castaneum*

There are numerous types of mandible within Mandibulata, but the shared character that links these diverse structural forms to define them as a functional mandible is the presence of the characteristic mandibular endite on the protopodite. This mandibular endite forms the biting edge. Therefore in order to study the development of the mandible it is necessary to choose a species that has this specialized structure present (Jenner, 2006). For this reason *Tribolium* is an appropriate model as it possesses a mandible with a typical gnathal edge differentiated into an incisor process and a molar process and is amenable to functional genetics.

Tribolium is an up and coming model organism, easy to culture and maintain in the laboratory and possessing a sequenced genome (Bucher et al., 2002; Bucher and Wimmer, 2005; Richards et al., 2008; Schroder et al., 2008). Whilst functional genetics in *Tribolium* may not be up to the level of genetic manipulation possible in the fruitfly, it is still at a level that surpasses the majority, if not all other arthropod species. Considerable work has been done on *Tribolium* embryogenesis, particularly in comparisons of developmental genes and gene regulatory networks that are of known importance in *Drosophila*. To date, there has been little study that has investigated the genes that are responsible for patterning the mandibular segment in a member of Mandibulata that unlike *Drosophila* possesses a typical mandible appendage. Importantly, then, *Tribolium* is a mandibulate that possesses a typical insect mandible and is amenable to functional genetic studies. There are inevitably significant differences between the mandible of *Tribolium* and the probable structure of the ancestral mandible of stem lineage mandibulates. As we have seen, the ancestral mandible has probably evolved from a maxilla-like precursor, and in many ways resembled the ancestral serial homologous biramous limb from which it was derived. The ancestral mandible was almost undoubtedly biramous and monocondylic (attached to the head with one condyle).

The *Tribolium* mandible, like all Hexapod mandibles, has lost both palps of the biramous limb structure and consists solely of an unsegmented protopodite. The *Tribolium* (hexapod) mandible is dicondylic, it has two attachment points to the head.

The defining characteristic of the ancestral mandible that distinguishes it from other appendages is the presence of a heavily sclerotized and developed endite which

is in the form of an incisor and molar biting edge, the *pars incisiva* and *pars molaris*. This well-developed endite that forms the biting edge on the protopodite is considered to be a homologous structure amongst Mandibulates (Edgecombe et al., 2003). The development of this structure is therefore of particular interest in understanding the mandible as a synapomorphy of Mandibulata.

Understanding the evolution of the mandible is not only interesting to understand the evolution of a clade-defining novel character, it is also necessary to understand the development of the ancestral mandible to form hypotheses about the evolution of derived gnathal appendages such as those mentioned above. For that purpose it is necessary to turn to an arthropod species that possesses a mandible.

After initial studies of mandible development in *Tribolium,* comparisons of mandible patterning genes in other taxa in interesting phylogenetic positions relative to *Tribolium* can be studied to determine whether significant aspects of mandible patterning are shared in diverse taxa. These data could then be used to either support or challenge the notion of mandible appendage and mandibular segment homology across Mandibulata.

Mandible patterning genes in *Tribolium*

Functional analysis has shown in *Tc Dfd* mutants there is a homeotic transformation of the mandible to an antenna[10] and a loss of the maxillary endite (Brown et al., 1999; Brown et al., 2000). The *Tribolium* orthologue of *cnc, Tc cnc,* has an expression pattern that is very similar to the expression pattern of *cnc* in *Drosophila* (Economou and Telford, 2009). Given that *cnc* is necessary to differentiate the mandibular segment from the maxillary segment in *Drosophila*, I wanted to investigate whether the mandible patterning function of *cnc* is conserved in other mandible-bearing mandibulates like *Tribolium*.

[10] Interestingly in *Drosophila Dfd* mutants there is a duplication of part of the cephalopharyngeal plates (most likely the vertical plate) which is derived from the procephalic lobe. If this derives from the antennal segment, it could indicate further similarity between the *Drosophila* and *Tribolium Dfd* mutant phenotype.

1.11 Introduction to results chapters

The introduction above has given some background to what is currently known about the evolution of the arthropod mandible. The mandible appendage has evolved from an ancestral biramous limb, which was serially homologous to all other post-antennal biramous limbs. The mandible probably evolved through a maxilla-like intermediate. The mandible gnathal edge, the putative synapomorphic character of the Mandibulata, is a modified endite on the protopodite (the base of the ancestral biramous limb). The gnathal edge is hypothesized to be on the proximal segment of the protopodite. This project aims to understand the evolution of the mandible from a leg, through a maxilla-like precursor in the phylum Arthropoda.

I am interested in addressing some specific evolutionary questions regarding the arthropod mandible. Are the mandibular segment patterning mechanisms homologous between different lineages of Mandibulata? Can mandibular appendage sub-structures be homologised to structures present on other appendage types? Does the mandibular patterning mechanism reflect the evolutionary history of the mandible from the protopodite of a biramous limb through a maxilla-like precursor?

In order to answer these questions, it is necessary to understand the molecular development of the mandible in at least one mandibulate arthropod. The primary aim of this research was to investigate mandibular segment and appendage patterning genes in a mandibulate arthropod. This is a necessary first step from which comparisons with other taxa can be made. As a representative of the Mandibulata and as a mandibulate arthropod with a primitive mandible, genes in the red flour beetle *Tribolium castanem* were studied to investigate their role in patterning the mandible. In particular the role of the homologue of *cnc,* which patterns the mandibular segment in *Drosophila,* was studied by RNA interference (RNAi) which is well characterized technique for *Tribolium* (Bucher et al., 2002).

In addition, the house spider *Achaearanea tepidariorum*, a chelicerate, was chosen to study mandible segment patterning genes in an outgroup to the hypothesized clade Mandibulata. From the results obtained from *Tribolium* and *Achaearanea,* it is hoped that some conclusions can be made about the conservation of mandibular segment patterning genes in mandibulates and the origin of mandible patterning functions of these genes.

I am interested in addressing several specific questions regarding the development of the arthropod mandible in *Tribolium*. Two structural features that are present on the developing mandible are the inner and outer lobes. The inner and outer lobes are frequently interpreted to represent respectively the developing molar and incisor processes of the mandibular gnathal edge. The incisor and molar processes have been alternatively hypothesized to be derived from one endite or from two endites.

I wanted to determine whether the outer and inner lobes correspond to the incisor and molar processes and whether these structures are derived from separate endites, or whether they derive from a single endite.

This question was investigated by detailed study of the expression of endite marker genes. The results of my investigations into questions about the mandibular endite are shown in chapter two.

I wanted to test whether the mandible gnathal edge is present on the proximal segment of the protopodite (the coxa), and whether there was any evidence for a division of the developing mandible into a subcoxa and coxa and if I could determine serial homology of these segments to segments of other gnathal appendages. I wanted to determine whether there was molecular evidence for the subcoxal derivation of the pleuron of the leg in order to determine any similarities that could suggest serial homology between mandible and leg appendage segments. The division of the mandible into subcoxa and coxa segments was studied by examining the expression of an appendage segment marker gene, the results of which are shown in chapter three.

cnc is necessary to pattern the mandibular segment of *Drosophila* and represses *Dfd* activity in this segment. To determine whether this function is conserved between the fruitfly and the beetle, the role of *Tc cnc* in patterning the mandibular segment and appendage in *Tribolium* was examined by RNAi. The effect of *Tc* cnc^{RNAi} on the expression of other genes was examined by *in situ* hybridisation. The results of these experiments are shown in Chapter four.

Tc Dfd has been shown to pattern the mandible and the base of the maxilla in *Tribolium,* and is therefore required to pattern the protopodite of these gnathal appendages. In chapter five I investigate this role in more detail by studying the effect of *Tc Dfd* knock down on other genes such as the PD domain genes, *notch* signalling

pathway and *Tc prd* in the maxillary appendage which have been shown to be activated by *Dfd* in *Drosophila.*

Chapter six will outline research on mandible patterning genes in the non-mandibulate *Achaearanea tepidariorum,* a member of Chelicerata. This species was chosen for study as an outgroup to the Mandibulata. This chapter will also briefly review what is known about orthologues of mandible patterning genes outside of Mandibulata.

Chapter 2: Development of the embryonic mandibular endite in *Tribolium*

2.1 Introduction

The *Tribolium* mandible is a modified endite attached to a protopodite, the remaining proximal part of the ancestral biramous limb. In this chapter I wanted to study the development of the mandibular endite in order to be able to compare this structure to the endites present on other appendages.

The incisor and molar processes together make up the functional biting edge of the mandible. This gnathal edge is the structure that differentiates the mandible from all other arthropod appendages. The mandibular gnathal edge is considered to be homologous between insects, crustaceans and myriapods (Kraus, 2001; Edgecombe et al., 2003).

The developing embryonic mandibular appendage is a relatively undifferentiated lobe-like structure with few morphological landmarks. Two structural features that are present on the mandibular lobe are the inner and outer lobes. The inner and outer lobes are frequently interpreted to represent the developing molar and incisor processes respectively. It has not been demonstrated whether the mandibular incisor and molar processes derive from the outer and inner lobes respectively. In addition, it has been suggested, but not convincingly demonstrated, that the inner and outer lobe are derived from two separate endites, as opposed to being derived from one endite which is the more conventional interpretation.

Therefore a detailed examination of the expression of genes that are expressed in the mandible endite was undertaken to answer these questions. The expression of appendage patterning genes that are expressed in the endites such as the PD domain gene *Tc dac* and an endite marker, *Tc prd,* was studied by *in situ* hybridization. The expression patterns of these genes was compared and related to morphological features of the developing mandible as determined by scanning electron microscopy.

The inner and outer lobes have been revealed by SEM studies of various hexapods (Machida, 2000; Liu et al., 2010; Oka et al., 2010). The inner and outer lobes are also present in the embryonic mandible of the millipede *Glomeris marginata* (Prpic and Tautz, 2003).

Expression of genes in the endites

The genes that control endite development are not known. As the mandible is a modified endite, these genes are very likely to be important for patterning the mandible. *Drosophila* does not possess endites in the larval appendages, but has a proximal region of the gnathal lobes that is interpreted to be homologous to the endites of other species (Jurgens et al., 1986). There are several genes which are known to be expressed in developing endites such as *prd* (Vanario-Alonso et al., 1995; Aranda et al., 2008), *dac* (Prpic et al., 2001; Prpic and Tautz, 2003; Ronco et al., 2008; Sewell et al., 2008) and Dll (Rogers et al., 2002). In addition to the role that *notch* plays in patterning appendage segments, preliminary results from the crustacean *Triops* indicate that *notch* is also involved in defining the endite boundaries in the phyllopodous limb (Sewell et al., 2008). The expression of *Tc ser* (a component of the *notch* signalling pathway) will be investigated in chapter 3.

The PD domain genes, *Dll, dac* and *hth* are expressed in endites but not in the manner in which they are expressed along the P/D axis of developing appendages. Therefore endite development does not involve a reiteration of the PD axis (Jockusch et al., 2004). A proximal domain of *Dll*, distinct from the distal telopodite domain is expressed in some endites. It has been suggested that *Dll* is involved in sensory organ development, such as chemoreceptors and mechanoreceptors (Mittmann and Scholtz, 2001; Prpic and Tautz, 2003). *Dll* expression is lacking from the insect mandible endite.

There are two domains of *Tc dac* expression, a proximal and distal expression domain. The proximal expression domain is expressed more strongly in the gnathal appendages than the trunk limbs. This proximal expression domain has been argued to be important for the development of the gnathal appendages. The proximal domain of *Tc dac* expression is strong in the mandible which may be indicative of its importance for mandibular development (Prpic et al., 2001). It has also been argued that the proximal domain of *dac* expression is ancestral to mandibulates and is important for patterning the endite lobes on all appendages of this clade (Sewell et al., 2008).

dac is expressed in the distal half of endites of the maxilla and labial appendages of numerous insect species (Prpic et al., 2001; Ronco et al., 2008) and the five endites of phyllopodous limbs of *Triops* and *Thamnocephalus* (Sewell et al., 2008). *dac* is associated with sclerotization in these species, the mandible as has been noted is a highly sclerotized endite (Sewell et al., 2008).

Aim of Chapter

This chapter will compare endite development in the mandibular and maxillary appendages of *Tribolium* by comparing the expression of genes in the mandibular and maxillary endites. The purpose of such an investigation is to determine whether the inner and outer lobes form the incisor and molar processes and whether the inner and outer lobes are formed from separate endites or from a single endite.

The expression of genes such as the PD domain gene *Tc dac,* the endite marker gene *Tc prd* and the segment polarity gene *Tc wg* will be studied in the developing endites. The morphology of the inner and outer lobes of the mandible will be studied by using scanning electron microscopy (SEM). The *pars incisiva* and *pars molaris* will be examined by microscopy of cuticle preparations of first instar larvae.

The question of whether the mandible gnathal edge (consisting of the incisor process and molar process) is formed from one or two endites will be investigated by comparing genes that are expressed in the mandibular endite to the maxillary endites. This question has implications regarding the serial homology of the mandibular endite to the maxillary endites. Machida hypothesized that the maxillary lacinia and galea are serially homologous to the incisor and molar processes respectively (see fig.2.1). Implicit in his hypothesis is that the incisor and molar processes develop from separate endites on the mandibular limb bud. If the mandible is derived from one endite, this would contradict any hypothesis that sought to homologize the incisor and molar processes to two separate endites.

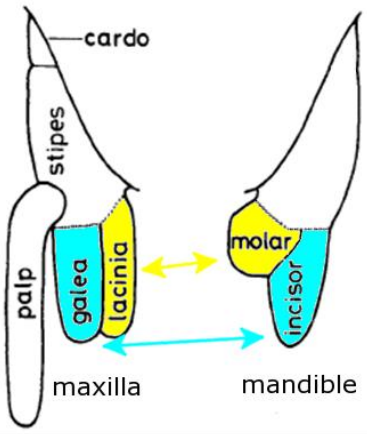

Fig.2.1. Hypothesis of the serial homology of the mandibular inner and outer lobes to the maxillary lacinia and galea endites after Machida (2000). The inner (yellow) and outer (blue) lobes of the mandible embryonic appendage are hypothesized to be serially homologous to the lacinia and galea by Machida. This hypothesis assumes that the molar and incisor processes are derived from the inner and outer lobes and the molar and incisor processes are derived from separate endites. If the molar and incisor processes are derived from one endite, then they can only be serially homologous to one of the maxillary endites. Figure is adapted from Machida (2000).

2.2 Results

Partial cDNA sequences of *Tc prd* and *Tc wg* were amplified and cloned in order to synthesize antisense labelled RNA probes to detect gene expression by *in situ* hybridization (see chapter eight). A clone of *Tc dac* was given courtesy of N. Prpic-Schäper.

Scanning electron micrographs of *Tribolium* embryos

Scanning electron microscopy (SEM) were taken of *Tribolium* to have higher resolution of mandibular morphology, to relate the inner and outer lobe found in other embryonic taxa to expression of the PD domain genes, endite. The developing mandible has two lobes perpendicular to the anterior posterior axis. The proximal lobe, the inner lobe, relates to the molar process and the distal lobe, the outer lobe, relates to the incisor process are distinguishable in the developing mandible (see fig.2.2).

Development of the mandibular endite as revealed by expression of *Tc prd*

Tc prd, in addition to its role as a pair rule gene, is expressed in the endites during embryogenesis (Aranda et al., 2008). There are two domains of expression in the maxilla that relate to the two endites, the presumptive lacinia and galea (fig. 2.3C,D). These two endites fuse to form the ventral branch (fig.2.3.D,E). There is one domain of expression in the mandible which is significantly larger than the domains found in either the maxillary or labial appendages. There is one domain of *Tc prd* expression in the developing mandible, at all stages of embryogenesis (fig.2.3B-E).

Tc prd expression is present in the developing endites soon after formation of the limb buds (fig2.3B). *Tc Dll* is expressed in all limb bud primordia except the mandible (fig.2.3A). *Tc Dll* is also expressed in the developing proximal maxillary endite, the lacinia endite (fig.2.3C,D).

In very late stages, immediately prior to the formation of the cuticle, the single domain of *Tc prd* expression can be seen to encompass the entire gnathal edge including the developing incisor and molar processes (see fig.2.4A-E). The morphology of these structures clearly resembles the structure of the mandible present in the first instar larva (compare fig.2.4A-E with fig.2.4H-J). This result suggests that the single domain of paired expression is a valid marker for the entire mandible endite that includes the incisor process and the molar process. *Tc prd* expression includes the developing

incisor (see arrow in fig.2.4E). The *Tc prd* expression domain does not appear to extend to the proximal boundary of the inner lobe (the developing molar).

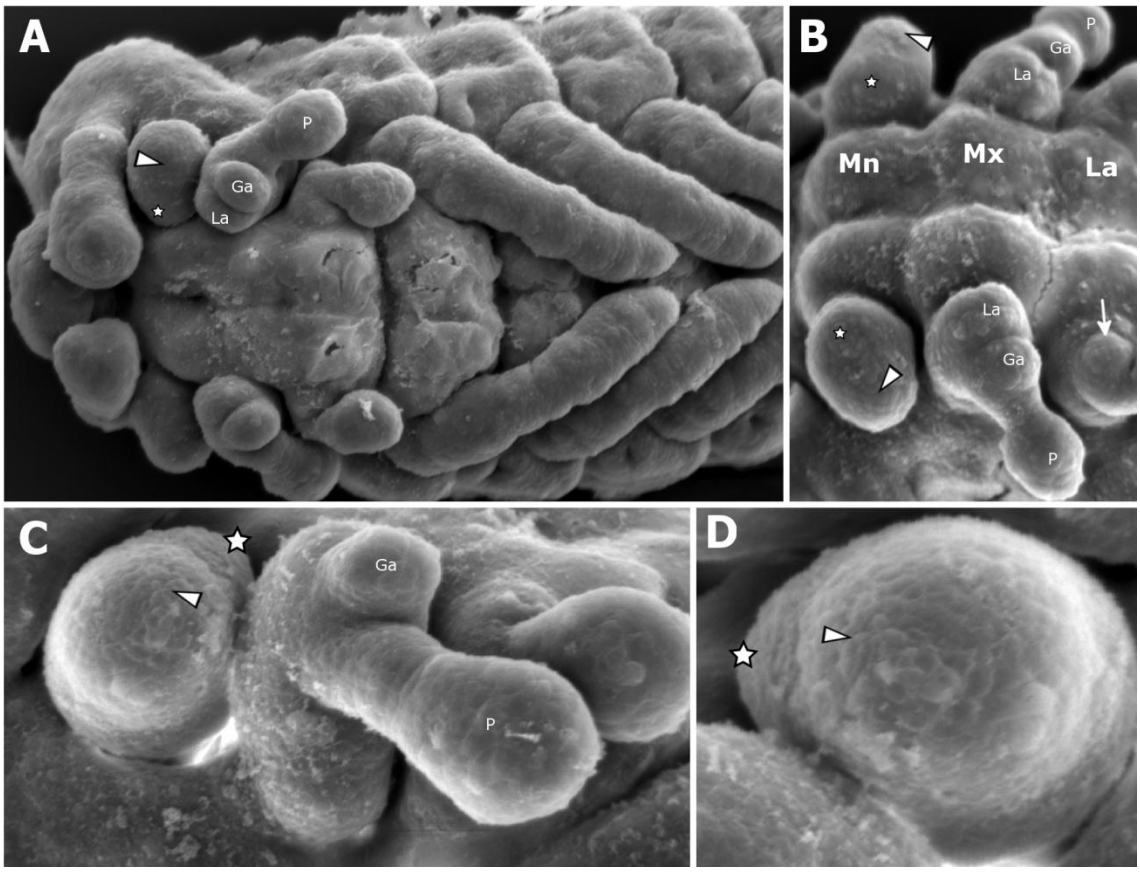

Fig.2.2. Scanning electron micrographs (SEMs) of developing gnathal appendages of *Tribolium* embryos showing the the inner and outer lobes. All views are ventral with anterior to the left unless otherwise indicated. Inner lobe is indicated with a star. The outer lobe is indicated with an arrowhead. The labial endite is indicated with an arrow in C. (A) Embryo at germ band retracting stage. Endites are visible on the maxillary appendage (La and Ga) and labial appendage (arrow). The labial appendages have not yet fused at the ventral midline. The inner and outer lobes of the mandible are faintly distinguishable. (B) (C) Lateral view of mandible, maxilla and labial appendages of an embryo at a similar stage to A. (D) Close up of mandibular limb bud with clearly distinguishable inner and outer lobes present. Anterior is bottom, lateral is to the right. Mandibular (Mn), maxillary (Mx) and labial (La) segments are indicated, as are the lacinia (Lc), galea (Ga), and maxillary palp (P).

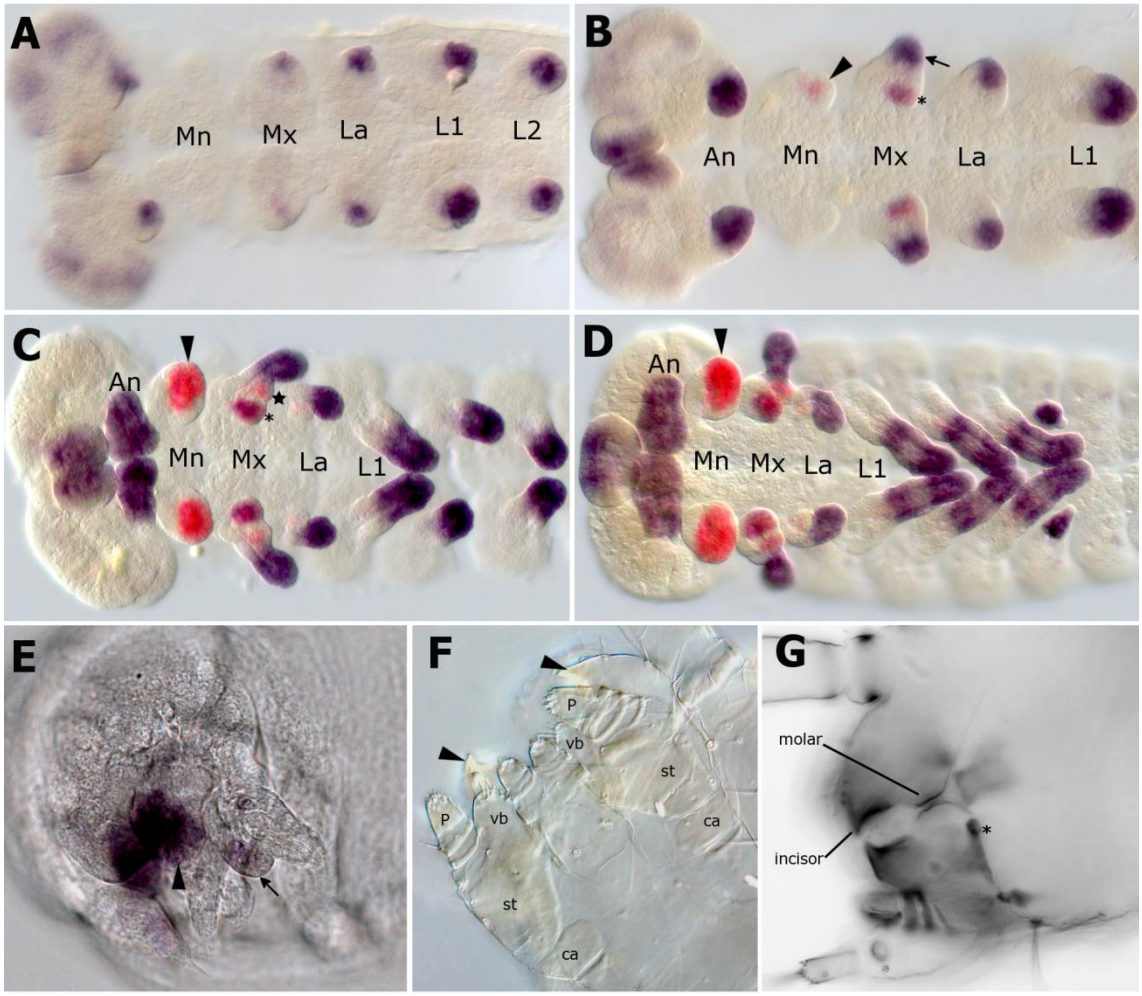

Fig.2.3. Development of the mandible and maxillary endites in the gnathal appendages of *Tribolium* embryos as revealed by expression of *Tc Dll* and *Tc prd.* All views are ventral with anterior to the left unless otherwise indicated. The mandible is indicated with an arrowhead. (A) Germ band extending stage embryo after formation of the limb buds, which are marked by *Tc Dll* expression. (B) Later germ band extending stage embryo. *Tc prd* expression is visible in the mandible. Expression of *Tc Dll* and *Tc prd* is present the maxillary lacinia endite (asterisk). *Tc Dll* is expressed in the maxillary palp (arrow). (C) Fully germ band extended stage embryo. Both maxillary endite lobes, the mandibular endites and the labial are marked with *Tc prd* expression. *Tc Dll* expression is present in the lacinia endite lobe (asterisk) but absent from the galea (star). The mandible has one domain of *Tc prd* expression which is significantly larger than either of the maxilla endite expression domains. (D) Germ band retracting stage embryo, Expression of *Tc prd* and *Tc Dll* is maintained in the same domains as in C. (E) Very late stage embryo prior to hatching as a larva with *Tc prd* detected by *in situ* hybridisation. *Tc prd* expression is present in the mandibular endites (arrowhead) and the maxillary endites (arrow). The lacinia and galea have fused to form the ventral branch (arrow). (F) Cuticle preparation of a first instar larva. The four segmented maxilla palp (P) is attached to the protopodite which consists of two segments, the cardo (ca) and stipes (st). The labial appendage consists of two appendages that have fused protopodites. (G) Mandibles of a first instar larva visualized by fluorescent microscopy. The mandible is unsegmented, with the gnathal edge consisting of an incisor process and molar process. One condyle of the mandible is visible (asterisk).

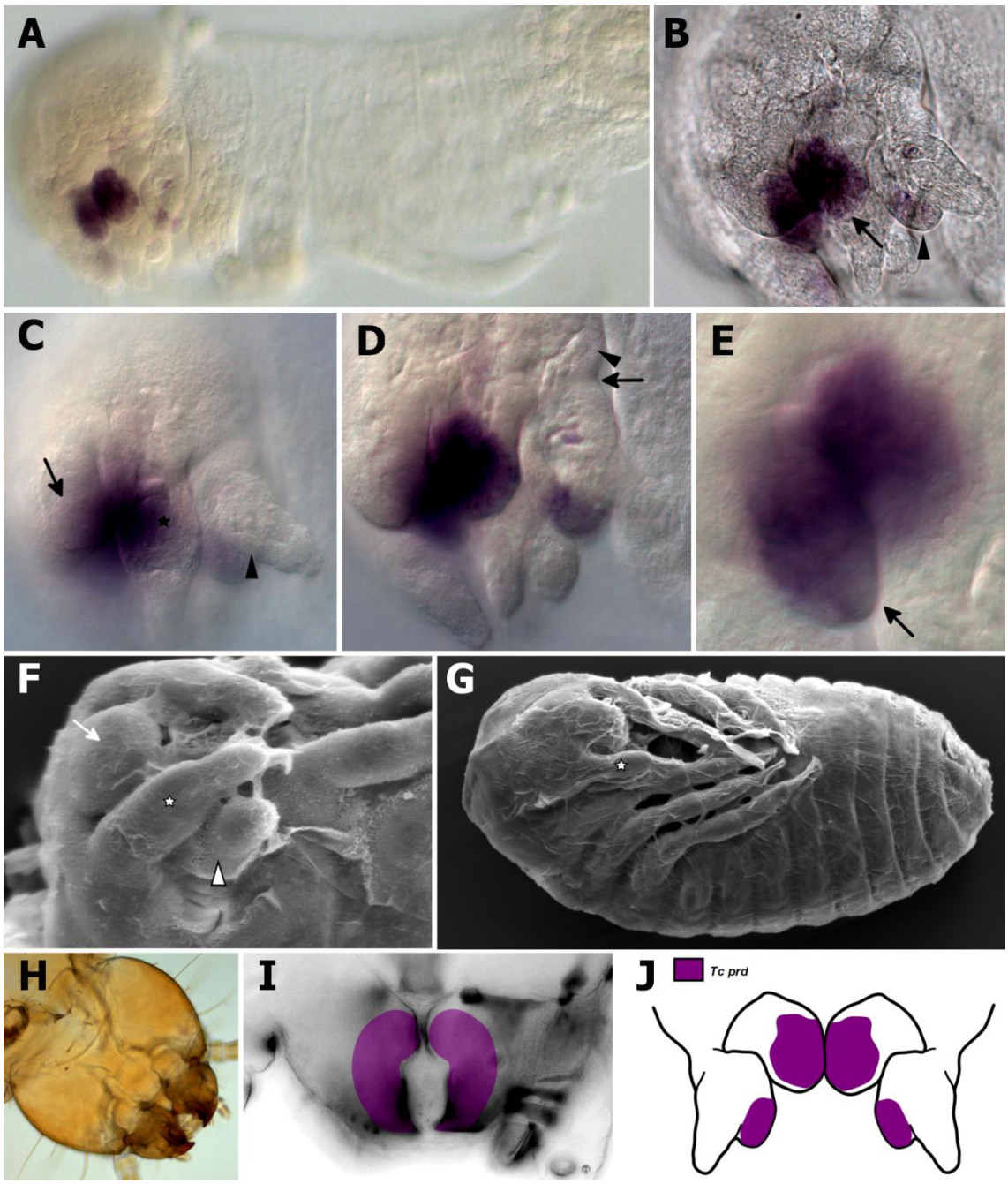

Fig.2.4. Expression of *Tc prd* in the mandibular endite relates the inner and outer lobe to the incisor and molar processes. The shape of the mandibular limb bud at this stage resembles the shape of the mandible in the larva. The distal tip of the mandible forming the incisor process and the area at the proximal limit of *Tc prd* expression forming the molar process. Labrum (arrow), maxillae (arrowhead) and antennae (star) cover the mandibles. Gene expression was detected by *in situ* hybridisation. (A-E) Expression of *Tc prd* in the mandible endite of a very late stage embryo prior to hatching. Anterior is left, dorsal is up. (A) View of entire larvae. (B-E) Different focal planes of A at higher magnification. (B) Gnathal appendages of A. Mandibular endite (arrow) and maxillary ventral branch consisting of fused lacinia and galea (arrowhead) are marked by *Tc prd* expression. (C) Antennae, labrum and maxilla are visible, mandible is visible as a purple blur (out of focus). (D) Possible cardo/stipes boundary (arrow), cardo is marked as an arrowhead. (E) Expression is visible in the pointed distal tip, the presumptive incisor process, of the mandibular endite. (F,G) SEM of late stage embryos. (H) Cuticle preparation of a fourth or later stage instar larval head. The mandible gnathal edge is more developed than the first instar larva shown in I. (I) mandibles of first instar larva with the expression domain of *Tc prd* highlighted on the gnathal edge based upon the position of the expression domain from A-E (J) Diagram outlining the shape of both the mandible and maxilla from A with *Tc prd* expression marked in purple. The expression of *Tc prd* is in one domain which encompasses the majority of the gnathal edge.

Comparison of the expression patterns of *Tc prd*, the PD domain genes, and *Tc wg* in the endites of the mandible and maxilla suggest the mandible is composed of one endite.

The expression of *Tc dac* in the mandible relative to *Tc prd* is similar to that seen in the maxilla endite lobes (fig.2.5). The proximal domain of *Tc dac* is expressed in the distal half of each maxillary endites whilst *Tc prd* is expressed throughout each endite lobe (fig.2.5C,F,G). In the mandible limb bud, *Tc dac* is expressed in the outer lobe with *Tc prd* expressed more or less continuously through both inner and outer lobes (fig.2.5A-C,E). The similarity of the relationship of *Tc dac* expression to *Tc prd* expression in the maxilla endites suggests that the mandible has only one endite (fig 2.5H).

Tc wg is expressed in a stripe that runs through the middle of the ectoderm of all appendages (fig 2.6A,C). *Tc wg* expression retracts from the endites of the maxilla in developing embryos to form two gaps in *Tc wg* expression. In the mandible, only one gap in *Tc wg* expression develops which suggests that there is only one endite present (fig 2.6B,D).

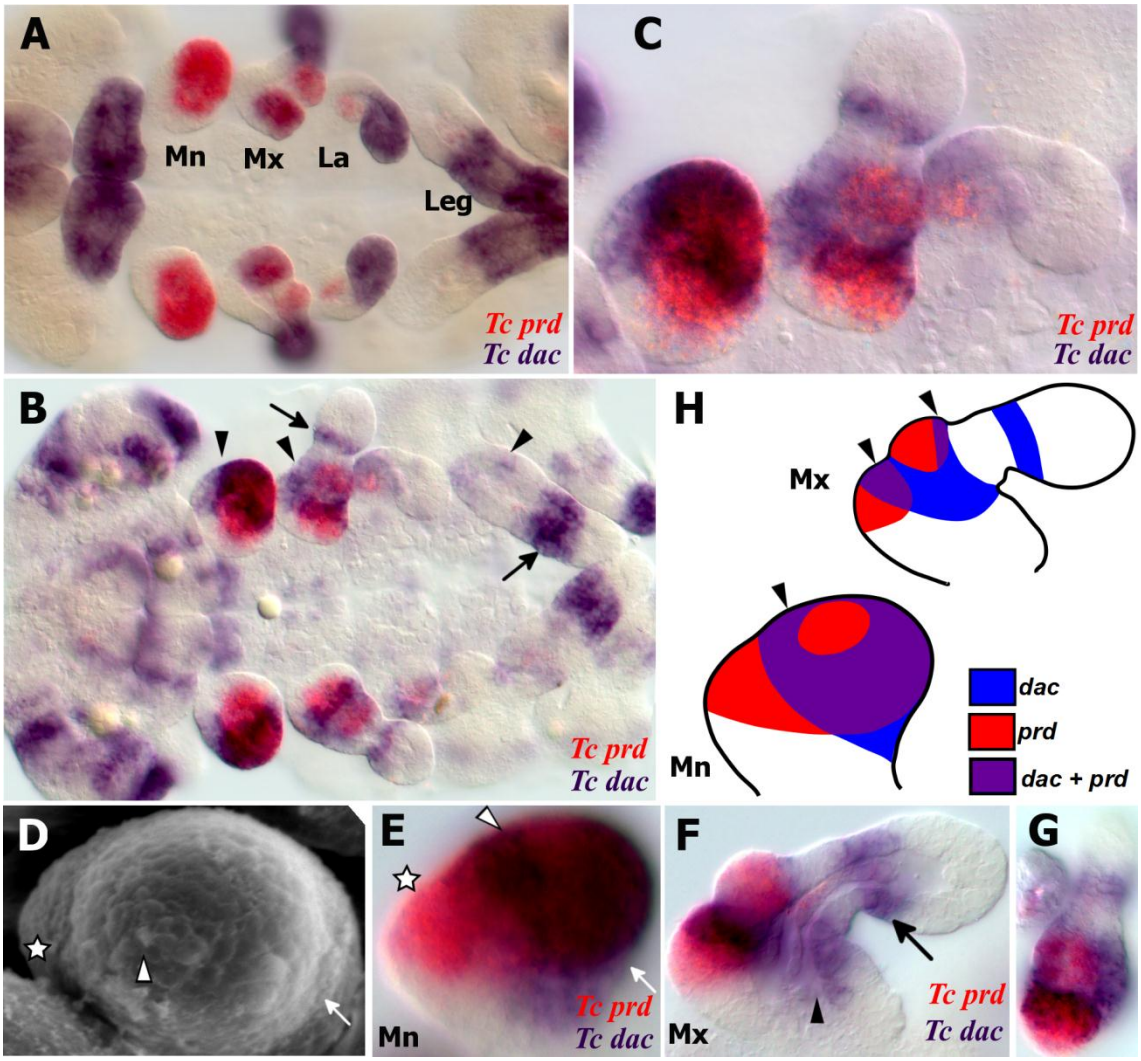

Fig 2.5. Expression of *Tc dac* and *Tc prd* in the mandible and maxillary endites. All views are ventral with anterior to the left unless otherwise indicated. Gene expression was detected by *in situ* hybridisation. (A) Expression of *Tc Dll* and *Tc prd* in a germ band retracting stage embryo. (B) Expression of *Tc dac* and *Tc prd* in a germ band retracting stage embryo. There are two domains of *Tc dac* expression, a proximal domain (arrowhead) and distal domain (asterisk). In the legs, the distal domain on *Tc dac* is more strongly expressed than the proximal domain. In the gnathal appendages, the proximal domain is more strongly expressed than the distal domain. The mandible domain of *Tc dac* is likely to represent the proximal domain (Prpic et al., 2001). (C) Gnathal appendages of B (D) SEM of mandible limb bud with inner lobe (star) and outer lobe (arrowhead) and subcoxa/coxa boundary (arrow) visible. Anterior is to the bottom, lateral is to the right. (E-G) dissected mandibles and maxillae from germ band retracting embryos. (E) Expression of *Tc prd* and *Tc dac* in a dissected mandible. Lateral is to the right, distal is to the top. (F) Expression of *Tc dac* and *Tc prd* in the mandible. (G) Expression of *Tc dac* and *Tc prd* in a dissected maxilla. Ventral view with anterior to the top. (H) Diagram illustrating the expression domains of *Tc dac* and *Tc prd* in the mandible and maxilla appendages. *Tc dac* is expressed in the distal half of the maxilla endites and the outer lobe of the mandible. *Tc dac* expression overlaps with *Tc prd* in the distal half of the endites (arrowheads). *Tc dac* expression is lacking from the proximal half of the endites and the inner lobe of the mandible. These expression patterns suggest that the mandible consists of one endite, the outer lobe of which is homologous to the distal half of the endites marked by *Tc dac* expression. The inner lobe is homologous to the proximal half which is marked by *Tc prd* but not *Tc dac* expression.

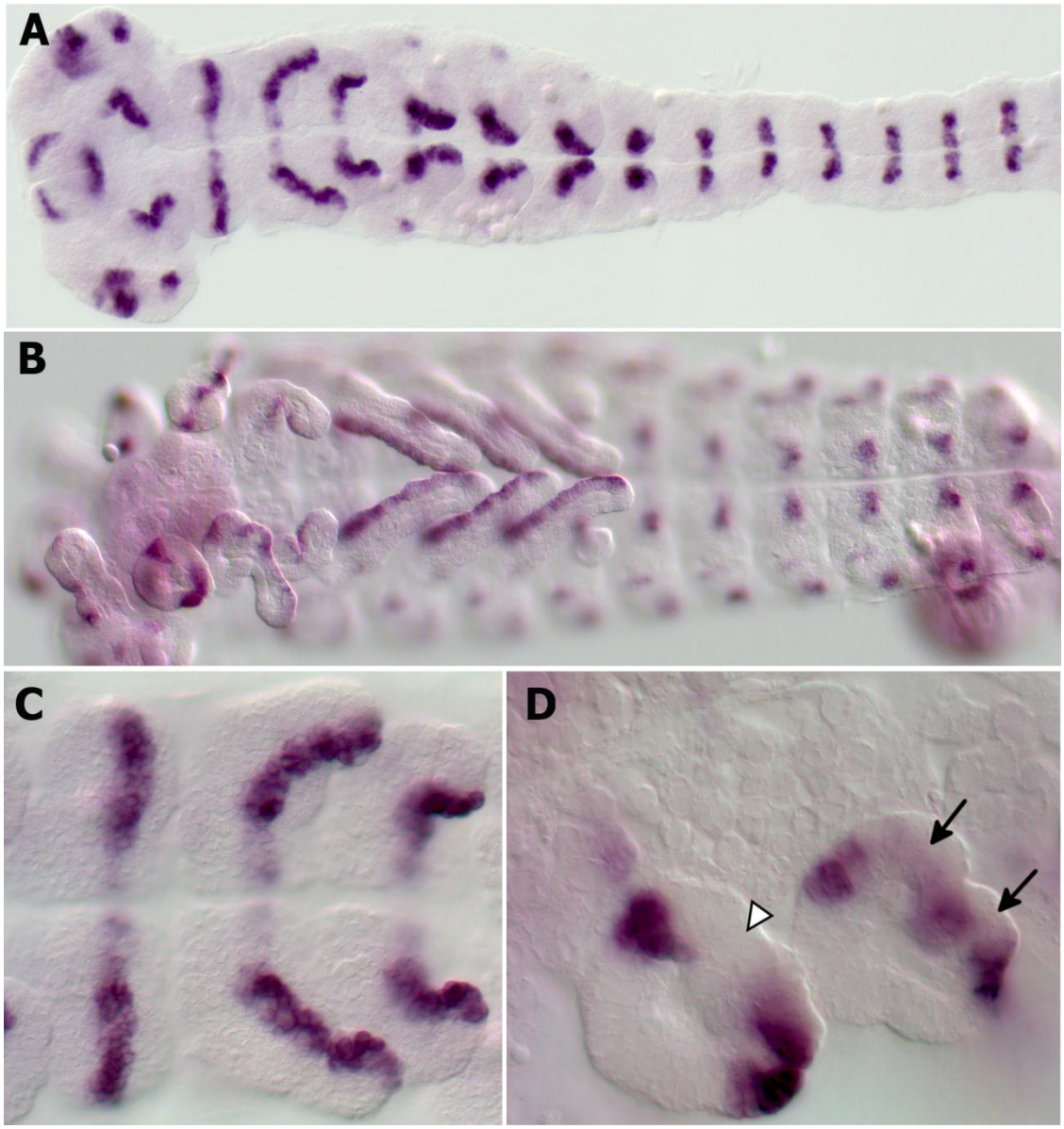

Fig.2.6. Expression of *Tc wg* in the mandible and maxilla suggest the the mandible has one endite. All views are ventral with anterior to the left. Gene expression was detected by *in situ* hybridisation. (A) Fully extended germ band stage embryo. Expression of *Tc wg* is in the posterior of each segment and in the medial region of each developing appendage. (B) Germ band retracting stage embryo. *Tc wg* expression is continuous in the medial region of the legs. In the gnathal appendages, there are spots of *Tc wg* expression in the ectoderm of the protopodite of the gnathal appendages with gaps of expression in the endites. (C) Close up of the gnathal appendages of the embryo shown in A. Expression is continuous throughout the medial part of each appendage including the developing endites. (D) Close up of the mandible and maxilla of the embryo. There is *Tc wg* expression in the ectoderm adjacent to the endites in the mandible and maxilla. *Tc wg* expression is lacking in the mandibular endite (white arrowhead) and the maxillary endites (arrows).

2.3 Discussion

Investigation of the expression of *Tc prd* in late stage *Tribolium* embryos shows that the inner and outer lobes that make up the mandibular endite develop into to the future incisor and molar processes. Study of the expression of the PD domain gene *Tc dac,* the endite marker *Tc prd* and *Tc wg* shows that the inner and outer lobes are derived from a single endite. The gnathal edge of the *Tribolium* mandible is therefore derived from one endite which disproves Machida's hypothesis that the mandible incisor and molar processes are derived from two separate endites which are homologous to the maxillary galea and lacinia.

The mandibular inner and outer lobe are the developing molar process and incisor process respectively

The developing mandibular appendage has two lobes which are visible in both SEMs (fig.2.2 and fig 2.5D) and *in situ* hybridizations of fixed *Tribolium* embryos (fig 2.5E). These two lobes are orientated perpendicularly to the anterior/posterior axis of the *Tribolium* embryo. The proximal or inner lobe relates to the molar process and the distal or outer lobe relates to the incisor process. The identity of these lobes has been suggested in other analyses.

This conclusion is confirmed in this study by the position of the expression domain of *Tc prd* in a late stage *Tribolium* embryo. Late *Tc prd* expression shows that the mandibular expression domain is in the developing incisor and molar processes (fig.2.4). The outer lobe is developing into the characteristic tooth morphology of the incisor process. Expression of *Tc prd* is present in the developing incisor process (fig. 2.4E) in late stage *Tribolium* embryos. The proximal limit of *Tc prd* expression matches to the developing molar process. This shows that *Tc prd* expression in the outer lobe relates to the developing incisor process and the inner lobe relates to the developing molar process.

Both gnathal edges marked by *Tc prd* expression, comprised of the developing incisor and molar processes, are migrating toward each other so that they are in contact at the midline in front of the mouth opening. This is characteristic of the orientation of the gnathal edge in the larva (fig 2.4H,I). In earlier stages of development, *Tc prd* expression is present throughout the inner and outer lobes (fig.2.5E).

The inner and outer mandible lobes seen in the embryonic *Tribolium* mandible are also found in other species and appear to be characteristic of the developing mandibular appendage. SEM studies in other insects have demonstrated that the inner and outer lobes are present and even more distinct in the cricket *Gryllus assimilis* (Liu et al., 2010), the sawfly *Athalia rosae* (Oka et al., 2010) and the jumping bristletail *Pedetontus unimaculatus* (Machida, 2000). A study into the expression of PD domain genes in the gnathal appendages of the millipede *Glomeris marginata,* shows that the mandible has an inner lobe and an outer lobe (Prpic and Tautz, 2003).

Comparison of *Tc dac* expression to the expression and function of *dac* in other mandibulates supports the conclusion that the outer lobe relates to the incisor process. *dac* expression is restricted to the outer lobe and expression appears to be strongest in a ring around the proximal part of the outer lobe adjacent to the inner lobe in the cricket *Gryllus* in a manner similar to that seen in *Tribolium* (Ronco et al., 2008). *Gm dac* expression in the millipede *Glomeris* is also more strongly expressed in the proximal part of the outer lobe (Prpic and Tautz, 2003).

In a functional study, knock down of *dac* in the adult Dung beetle resulted in deletion of the region in between the incisor and molar processes. The adult mandibles in this species are characterised by an elongated incisor process. (Simonnet and Moczek, 2011). This result is consistent with the expression domain of *Tc dac* in the *Tribolium* mandible in the proximal part of the outer lobe in between the developing molar and incisor processes. Although unpublished functional work performed in *Tribolium* has not shown a role for *Tc dac* in patterning the mandible (Simonnet and Moczek, 2011), this does not necessarily detract from conclusions based on its use as a marker for the mandibular gnathal edge.

The mandibular gnathal edge is derived from one endite

Evidence provided from several genetic markers supports the conclusion that the mandible possesses one endite. Using *Tc prd* as a marker for endite development reveals the presence of one domain of *Tc prd* expression in the mandible compared to two domains in the maxilla. This suggests that there is only one endite present in the mandible. This conclusion contradicts the hypothesis of Machida (2000) which posits that the mandible incisor process and the molar process are homologous to the galea and lacinia maxillary endites, as it implies they are derived from two endites.

A previous study of the *Tc wg* expression pattern in *Tribolium* has suggested that the mandible consists of one endite (Jockusch et al., 2004). *Tc wg* is expressed in a stripe that runs through the middle of the ectoderm of all appendages. *Tc wg* expression retracts from the endites of the maxilla to form two gaps in *Tc wg* expression. In the mandible, only one gap in *Tc wg* expression develops which suggests that there is only one endite present (fig.2.6D).

Comparison of the expression of *Tc dac* relative to *Tc prd* expression shows similarities of the mandibular endite to both of the maxilla endites. The co-expression of *Tc dac* and *Tc prd* in the mandibular endite resembles the co-expression of these genes in both maxillary endites. *Tc dac* expression overlaps with the distal half of *Tc prd* expression in developing endites (arrowhead in fig 2.5H). *Tc dac* is expressed in the distal half of the maxillary endites whilst *Tc prd* is expressed throughout both maxillary endites. In the mandible limb bud, *Tc dac* is expressed in the outer lobe with *Tc prd* expressed more or less continuously through both inner and outer lobes. The similarity of the relationship of *Tc dac* expression to *Tc prd* expression in the maxilla endites therefore supports the conclusion that the mandible has only one endite.

This expression pattern of *dac* in the distal half of endites appears to be conserved across mandibulates. The expression of *dac* in the distal half of endites is observed in the cricket *Gryllus* (Ronco et al., 2008). Expression of *Dac* in the phyllopodous limbs of *Triops* is restricted to the distal half of the developing endites (Sewell et al., 2008). Expression of *dac* is not observed in the distal half of endites in chelicerates (Prpic and Damen, 2004). However, chelicerate endites are not hypothesized to be homologous to mandibulate endites as they derive from unsegmented protopodites as discussed in chapter one (Boxshall, 2004; Sewell et al., 2008).

As there is only one mandibular endite that divides into two lobes that develop into the molar and incisor processes, the mandibular endite has evolved from a typical lobe-like endite to form an endite consisting of two lobes. The mandible endite has therefore expanded proximally to form the molar process (inner lobe) and distally to form the incisor process (outer lobe). The outer lobe is derived from the distal half of the endite (marked by *Tc dac* and *Tc prd* expression), and the inner lobe is derived from the proximal half of the endite which is lacking *Tc dac* expression and is marked by *Tc prd* expression.

Endite patterning mechanism

The expression domain of both *Tc dac* and *Tc prd* in the endites suggest a possible functional role for these genes in patterning the gnathal appendage endites of *Tribolium*, and possibly other arthropods. The functional role of *Tc prd* and *Tc dac* to pattern the maxillary endites was tested by knocking down by parental RNAi. However, neither experiment was successful in determining an endite patterning role of these genes (see appendix 5). It would be interesting to investigate the expression of homologs of *prd* to see if *prd* expression is conserved in the endites of other arthropods.

The gnathal edge of the mandible is the structure that differentiates the mandible from all other arthropod appendages. This chapter has shown, that the gnathal edge is derived from the inner and outer lobes which themselves derive from one endite. One gene, *Tc cnc,* that differentiates the mandibular segment from the maxillary segment in *Tribolium*, including the mandibular endite from maxillary endite identity, will be investigated in chapter four. Another gene that is required to pattern mandibular and maxillary endites, *Tc Dfd,* will be investigated in chapter five.

Chapter 3:
Division of the *Tribolium* embryonic mandible into a subcoxa and coxa

3.1 Introduction

In this chapter I am interested in the structure of the protopodite of the *Tribolium* mandible, specifically if there is any evidence of segmentation. The ancestral biramous limb was composed of two rami, an exopodite-derived ramus and a telopodite-derived ramus, which were connected to the protopodite which is located at the base of the appendage. The insect mandible has evolved from a biramous limb by losing both palps. The structure that remains is therefore the remaining protopodite complete with the gnathal edge, the character that distinguishes a mandible from other appendage types. I was also interested in the structure of the protopodites of other post-antennal appendages. By comparing the mandibular protopodite to the protopodites of other appendages it may be possible to show similarities that are suggestive of homology.

The hexapod mandible is an unsegmented appendage in both the larva and the adult. However, it has been observed in the developing mandible of various hexapods that there is a so-called subcoxa/coxa division (Machida, 2000; Liu et al., 2010; Oka et al., 2010). It has also been hypothesized that these subcoxal and coxal segments in the mandible are homologous to segments in the maxillary appendage, the cardo and stipes respectively (see fig.3.1A) (Machida, 2000).

In order to examine the possible existence of a segmental subdivision in the developing embryo, a segmental marker was used to study the developing mandibular appendage. The Notch signalling pathway is involved in the formation of arthropod limb segments and is important in the development of arthropod appendages. There has been no study of the Notch signalling pathway in the gnathal appendages to date. To determine the precise identity of each appendage segment in the developing embryo, expression of the PD domain genes was studied in conjunction with the Notch signalling pathway.

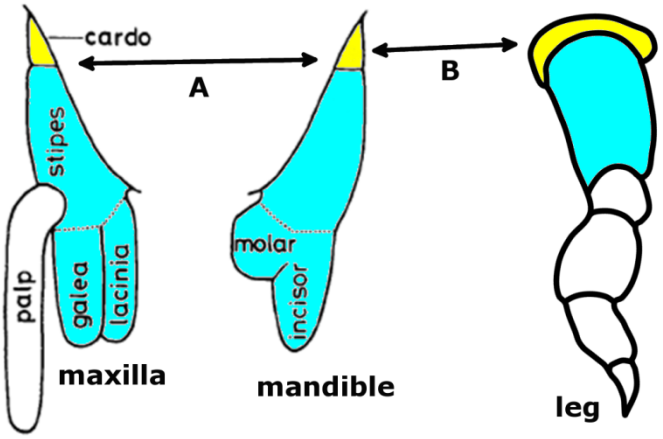

Fig.3.1 Hypotheses of serial homology of the putative mandibular subcoxa to the cardo of the maxilla and the subcoxa of the leg. Possible homologous appendage structures are indicated with the same colour. Figure is modified from Machida (2000). Subcoxal segments are shown in yellow, distal protopodite segments are shown in blue. (A) Machida has hypothesized that the mandible is divided into a subcoxa (yellow) and coxa (blue) which is serially homologous to the cardo (yellow) and stipes (blue) of the maxilla. (B) If there is a subcoxal segment present in the leg (yellow), there is a possibility to homologize it to other appendage segments, in this example the leg subcoxa is homologized to the putative subcoxa of the mandible.

Appendage segments and the Notch signalling pathway

One clade defining character of the phylum Arthropoda is the presence of limb segments. It has been shown that the Notch signalling pathway is responsible for the creation of leg segment joints in *Drosophila.* The Notch signalling pathway is often responsible for setting up cell boundaries and defining populations of cells (Bray, 2006).

For the purposes of a genetic marker for segment boundary formation I chose to study the expression of the *Tribolium* homologue of the gene *serrate (Tc ser). Tc ser* was significantly easier to detect by *in situ* hybridization than *notch* (data not shown). *ser* is a transmembrane ligand of the Notch receptor and regulates Notch activation in adjacent cells. *ser* is expressed in a ring of cells that are on the distal part of each segment. *notch* is expressed in a ring of cells that form the joint between leg segments and is expressed immediately adjacent to cells expressing *ser. ser* is therefore expressed slightly proximally to *notch* and to where the segment boundary will form (Rauskolb, 2001).

The Notch signalling pathway is involved in the formation of leg segments in *Drosophila* and is regulated by the PD domain genes. For example the first ring of *ser*

expression in *Drosophila* relates to the coxa and is activated by *hth* within the presumptive coxa. *Dll* represses *ser* in the presumptive telopodite at this stage (Rauskolb, 2001; de Celis Ibeas and Bray, 2003; Hao et al., 2003; Greenberg and Hatini, 2009). Therefore, it is possible to relate PD domain gene expression to precise appendage segments in developing embryos by comparing the expression domains of the PD domain genes with *notch* rings of expression that relate to specific segment boundaries.

Expression of *notch* in a segmental fashion in the leg appendages of the spider *Cupiennius salei* reveals that it is likely that Notch mediated definition of segment boundaries is also present in chelicerates and likely to be conserved across Arthropoda (Prpic and Damen, 2009).

PD domain genes are conserved across Arthropoda in all appendage types

The expression and function of PD domain genes are conserved across arthropod leg development (Angelini and Kaufman, 2005). The expression of PD domain genes in gnathal appendages has been shown to be similar to that observed in the developing legs. The expression patterns of PD domain genes has been studied in the gnathal appendages in several species with mandibulate mouthparts (Abzhanov and Kaufman, 2000b; Beermann et al., 2001; Prpic et al., 2001; Inoue et al., 2002; Rogers et al., 2002; Prpic et al., 2003; Prpic and Tautz, 2003; Jockusch et al., 2004; Mito et al., 2008; Prpic and Telford, 2008; Ronco et al., 2008; Liubicich et al., 2009). In all studied species the expression domains of PD domain genes are conserved along the proximal-distal axis between gnathal appendages and leg appendages.

The mandible is a notable exception in species that are lacking a palp of telopodite origin. These mandibles lack the telopodite *Dll* expression domain which is strong evidence for the gnathobasic nature of the mandible appendage (Niwa et al., 1997; Popadic et al., 1998; Scholtz et al., 1998).

Proximal domain of *Tc dac*

There are two domains of *Tc dac* expression, a proximal and distal expression domain. The proximal expression domain is expressed more strongly in the gnathal appendages than the trunk limbs. This proximal expression domain has been argued to be important for the development of the gnathal appendages (Prpic et al., 2001). The proximal domain of *dac* expression is strong in the mandible which may be indicative

of its importance for mandibular development (Prpic et al., 2001). Prpic *et al.* have argued that the proximal domain of *Tc dac* is evidence of serial homology of the whole mandible to the coxa of the leg (Prpic et al., 2001).

It has also been argued that the proximal domain of *dac* expression is ancestral to mandibulates and is important for patterning the endite lobes on all appendages of this clade (Sewell et al., 2008).

Numerous studies have investigated the expression patterns of PD domain genes in diverse taxa, but the relationship between the PD domain genes and notch signalling has only been investigated in *Drosophila*. There has been no study of the Notch signalling pathway in a mandibulate arthropod with segmented gnathal larval appendages.

Aim of Chapter

By studying the expression of the segmentation marker *Tc ser*, it was hoped that several specific questions about the structure of the embryonic mandible and serial homology of particular mandibular structures could be addressed. Most importantly, I wanted to discover if there was any evidence of segmentation in the embryonic mandibular appendage. Machida and others have suggested through SEM studies of a bristletail that the embryonic mandible is divided into a subcoxa and coxa (Machida, 2000; Oka et al., 2010).

Also, I wanted to investigate serial homology of the arthropod mandible protopodite to other appendages. If there is evidence of segmentation in the mandible, by studying the co-expression of *Tc ser* and the PD domain genes it is possible to find similarities that are evidence of homology of mandibular structures to other appendage structures.

There were specific questions of serial homology that I wanted to address. Machida and Kukalova-Peck have both hypothesized that the subcoxa of the embryonic mandible is serially homologous to the cardo of the maxilla (Machida, 2000; Haas et al., 2001). Machida has also hypothesized that the coxa of the embryonic mandible is homologous to the stipes of the maxilla (shown in fig. 3.1A).

I also wanted to investigate the subcoxal hypothesis of the leg pleuron. The pleuron is defined as the part of the body where the legs attach to the thorax (Snodgrass, 1935; Deuve, 2001; Boxshall, 2004; Grimaldi and Engel, 2005). In the

majority of insects, the coxa is attached to separate sclerites which are hypothesized to have subcoxal origin (Snodgrass, 1935; Boxshall, 2004). Boxshall has commented that if the pleuron of the leg has a subcoxal origin then it is possible to homologize protopodite segments of the leg to other appendages (Boxshall, 2004). The presence of a leg subcoxa could be revealed by the expression of *Tc ser.* In which case, evidence for the homology of the leg subcoxa to other segments can be shown (see fig.3.1B).

The expression of the PD domain genes, limb segmentation genes and an endite marker, *Tc prd,* was studied by *in situ* hybridization. The expression patterns of the PD domain genes was compared to the expression of the Notch signalling pathway in order to determine the precise segmental affinity of the PD domain gene expression patterns. In addition the morphology of the developing appendages will be studied using SEM of embryos and microscopy of cuticle preparations of first instar larvae. By comparing expression of these genes in different appendages, evidence for serial homology of any mandibular structures to other appendage structures for example the cardo and stipes on maxilla, or the subcoxa of leg will be considered.

3.2 Results

Partial cDNA sequences of *Tc ser, Tc Dll, Tc hth* and *Tc prd* were amplified and cloned in order to synthesize antisense labelled RNA probes to detect gene expression by *in situ* hybridization (see chapter eight). A clone of *Tc dac* was given courtesy of N. Prpic-Schäper.

Scanning electron micrographs of *Tribolium* embryos

SEMs were taken of *Tribolium* to have higher resolution of mandibular morphology, to relate the subcoxa/coxa boundary found in other embryonic taxa to expression of the PD domain genes, endite and segmentation marker genes.

A groove can be seen on the lateral side of the developing mandible (see fig.3.2). This groove is interpreted here as representing a subcoxal/coxal segment boundary that is only visible during embryogenesis. A groove is also present at a similar position in the developing maxilla (see fig.3.2A,C-D) and labial appendage (see fig.3.2D). The developing maxilla undergoes complex morphological changes during embryonic development. The orientation of the palp the protopodite changes during embryogenesis.

Tc ser expression demarcates appendage segments in all appendages

In order to mark the development of segments, the *Tribolium* homolog of *serrate, Tc ser,* expression was detected in the developing appendages of *Tribolium* embryos. *Tc ser* expression is complex, with numerous expression domains in different embryological structures (fig.3.3A,C). *Tc ser* is expressed in rings along the proximal-distal axis of all appendage types (fig.3.3, fig.3.4). *Tc ser* expression was located immediately proximal to the developing limb joints in the distal part of each limb segment (fig.3.10A,F). The situation is more complex in the gnathal appendages, as the morphology of these appendages is less uniform than the telescopic-like appearance of the leg appendages. These appendage domains of *Tc ser* relate to grooves revealed by SEM (fig.3.3). The orientation and morphology compounded with the relative faintness of some of the expression domains means that dissection of gnathal appendages is required to study *Tc ser* expression.

In the leg there are five rings of expression of *Tc ser* (see fig.3.3A,C). The identity of each of these domains was determined by comparison of *Tc ser* expression

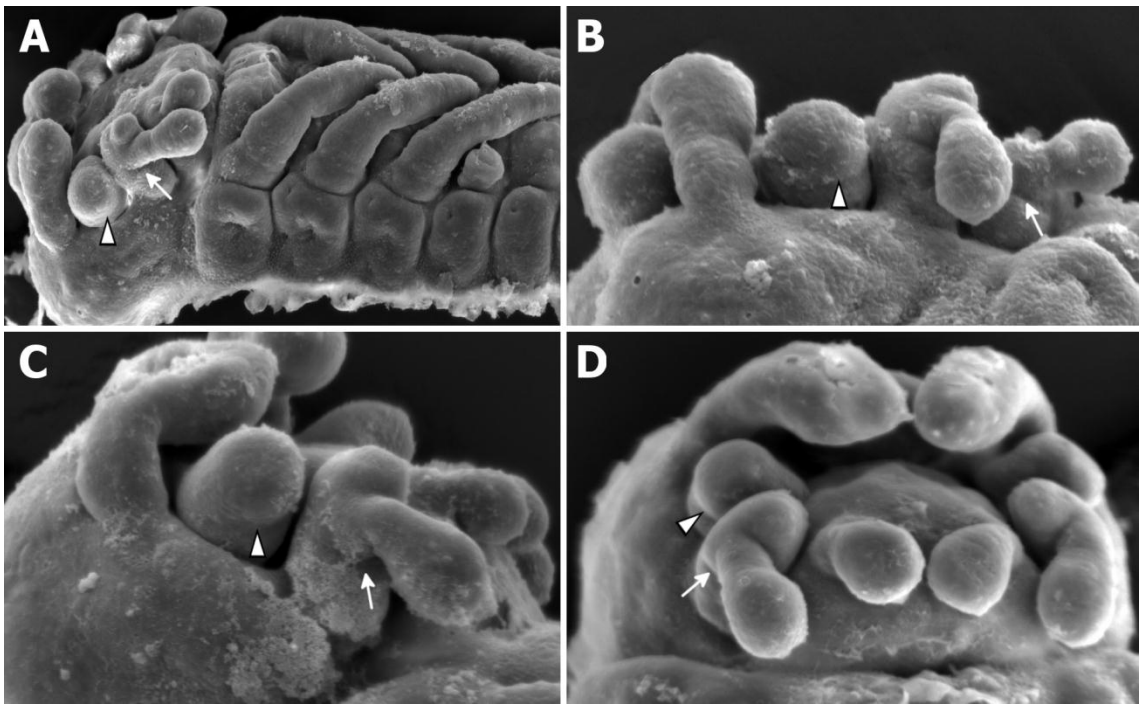

Fig.3.2. Scanning electron micrographs (SEMs) of developing gnathal appendages of *Tribolium* embryos showing the presence of a subcoxa/coxa boundary on the developing mandible. All views are lateral with anterior to the left unless otherwise indicated. Subcoxal/coxal division on the mandible is indicated with an arrowhead. (A) Embryo at germ band retracting stage, ventral-lateral view. Proximal division that may relate to future cardo/stipes segment boundary on the maxilla is indicated with an arrow. (B) Germ band retracting embryo at an earlier stage than A. Labial appendages have not begun to fuse, a subcoxa/coxa division is visible on the labial appendage (arrow) and the mandible. (C) Close up of head of an embryo during dorsal closure. (D) Ventral view of head of germ band fully retracted embryo. Anterior is to the top. The labial appendages have begun to fuse to form a lower 'lip' below the mouth opening. A lateral groove is present on the protopodite of the mandible and maxilla.

domains with *Tc prd* expression (fig.3.4B-D), PD domain gene expression (fig.3.5A-K) and appendage morphology in late stage embryos (fig.3.10). The developing appendages of late stage embryos are more developed and resemble the morphology of appendages of first instar larvae. The similar identity of the subcoxal and coxal domains of *Tc ser* was also determined by studying the early expression of these domains with *Tc dac* as a marker (fig.3.8 and fig.3.9).

The five rings of expression of *Tc ser* correspond to the distal boundaries of the following leg segments in proximal to distal order: subcoxa-1, coxa-2, trochanteral-3, femur-4, tibia-5 (see fig.3.5I-L and fig.3.10A-C,E-F). The tarsal segment is more distal to the tibial *Tc ser* domain. Similarities of expression revealed by comparison of the leg *Tc ser* domains to the gnathal appendage *Tc ser* domains, particularly the leg subcoxal-1 and coxal-2 domains, has led me to use the same numerical indications to describe the gnathal appendage domains. This nomenclature will also aid in descriptions of the

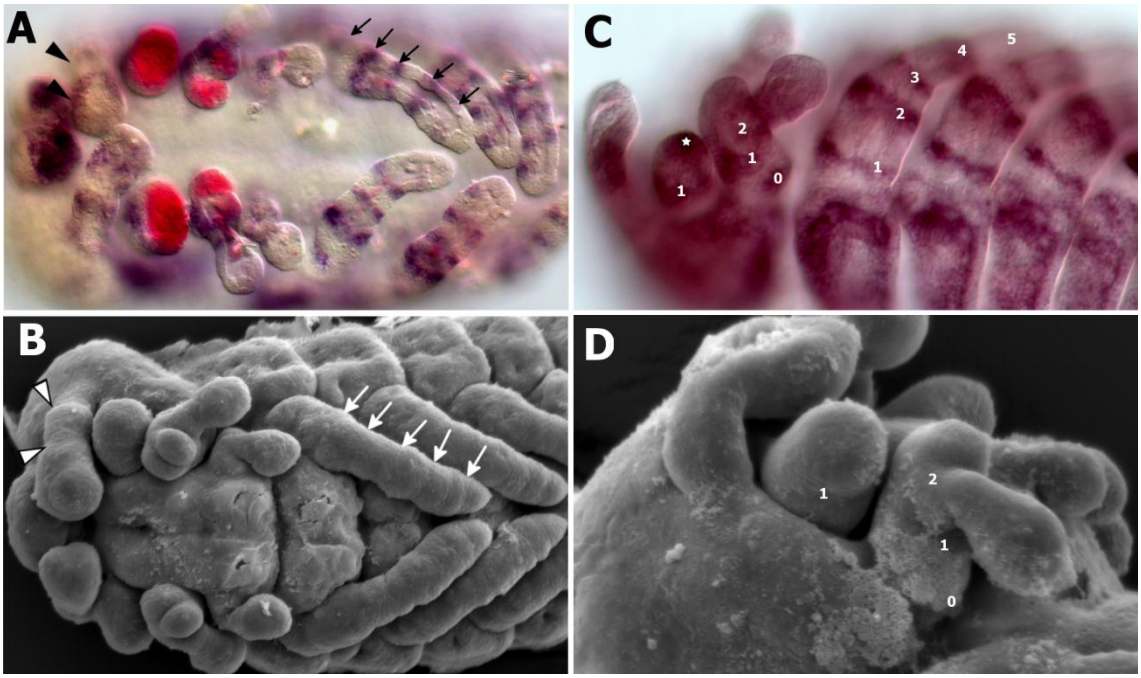

Fig.3.3. *Tc ser* expression domains mark the position of developing appendage segments and shows that there is a mandible subcoxal segment. All views are ventral with anterior to the left unless otherwise indicated. Gene expression was detected by *in situ* hybridisation. (A) *Tc ser* (blue) and *Tc prd* (red) expression in a fully retracted stage embryo. The five ring domains of *Tc ser* expression are marked by arrows. There are two ring domains in the antenna (arrowheads). *Tc ser* domains are present in the mandible and maxilla but are not readily distinguishable. (B) SEM of an embryo at a similar stage of embryogenesis to A. The position of the developing segmental boundaries, visible as grooves perpendicular to the P/D axis of the appendages, are in the same positions as the ring domains of *Tc ser* visualized in A. The segmental grooves in the legs are marked by arrows, in the antenna by arrowheads. (C-D) Higher magnification of gnathal appendages. Numbers indicate *Tc ser* domains. (C) Expression of *Tc ser* (blue) in an embryo during dorsal closure. There are two domains of expression in the mandible limb bud. A distal spot (star) domain that is in the outer lobe and a ring domain-1 of *Tc ser* that marks the division of the subcoxa and coxa. The maxilla has several domains of *Tc ser* expression. In the protopodite, there are two ring domains numbered 1 and 2 and a smaller more proximal stripe domain, numbered 0. (D) SEM of an embryonic head at a similar stage to that of C. The subcoxa/coxa boundary is visible in both the mandible and maxilla in a similar position to the subcoxal domain-1 of *Tc ser* expression visible in C.

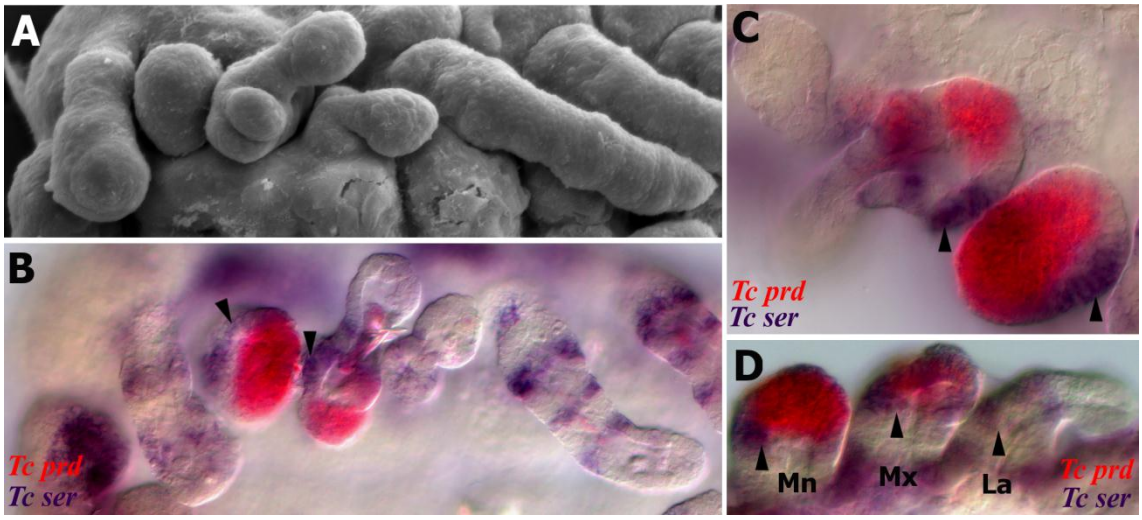

Fig.3.4. Expression of *Tc prd* relative to *Tc ser* suggests that endites develop in the distal-most segment of the mandible, maxillary and labial protopodites. All views are ventral with anterior to the left unless otherwise indicated. The subcoxal domain of *Tc ser* is marked with an arrowhead. Gene expression was detected by *in situ* hybridisation. (A) SEM of developing appendages of a fully retracted *Tribolium* embryo. (B-D) *Tc ser* (blue) and *Tc prd* (red) expression embryos of a similar stage to A. *Tc prd* is visible in the mandible, maxilla and labial endites. (B) The mandibular *Tc prd* domain is clearly more distal to the ring domain of *Tc ser*. The orientation of the maxilla and labial appendages prevent clear visualization of the relative expression of *Tc ser* and *Tc prd.* (C) Close up of gnathal appendages of an embryo at a similar stage to those in A and B. The subcoxal domain of *Tc ser* expression (arrow) is more proximal than the *Tc prd* endite domains. (D) Lateral view of gnathal appendages. Expression of *Tc prd* is distal to the *Tc ser* subcoxa ring domain in the mandible, maxilla and labial appendages.

expression domains of these genes. The evidence for the similarity of the appendage *Tc ser* domains between the leg and gnathal appendages is the similarities observed in *Tc prd* expression (see fig.3.4) PD domain gene expression (see fig.3.5) and early *Tc ser* expression marked by *Tc dac* (see fig.3.8 and fig.3.9).

The expression of *Tc ser* in the maxilla was the most difficult to interpret and required dissection to identify expression domains. At reasonably late stages of development, prior to dorsal closure and fusion of the labial appendages, there are two domains in the protopodite, the subcoxal-1 and coxal-2 domain, and at least two ring domains in the palp (see fig.3.5D,E and fig.3.7A,E,F). There was a fifth distal ring domain that was detected in one dissected maxilla (fig.3.5G). The expression of this ring domain was particularly faint. The maxillary subcoxal-1 domain of *Tc ser* is clearly associated with a cell boundary (see fig.3.7E,F). In late stages of maxilla development, there is the appearance of a stripe or spot of *Tc ser* at the base of the appendage proximal to all the other rings of *Tc ser* expression (see fig.3.7A,C). The significance of this proximal-0 *Tc ser* domain, and whether it relates to the future cardo/stipes is not known.

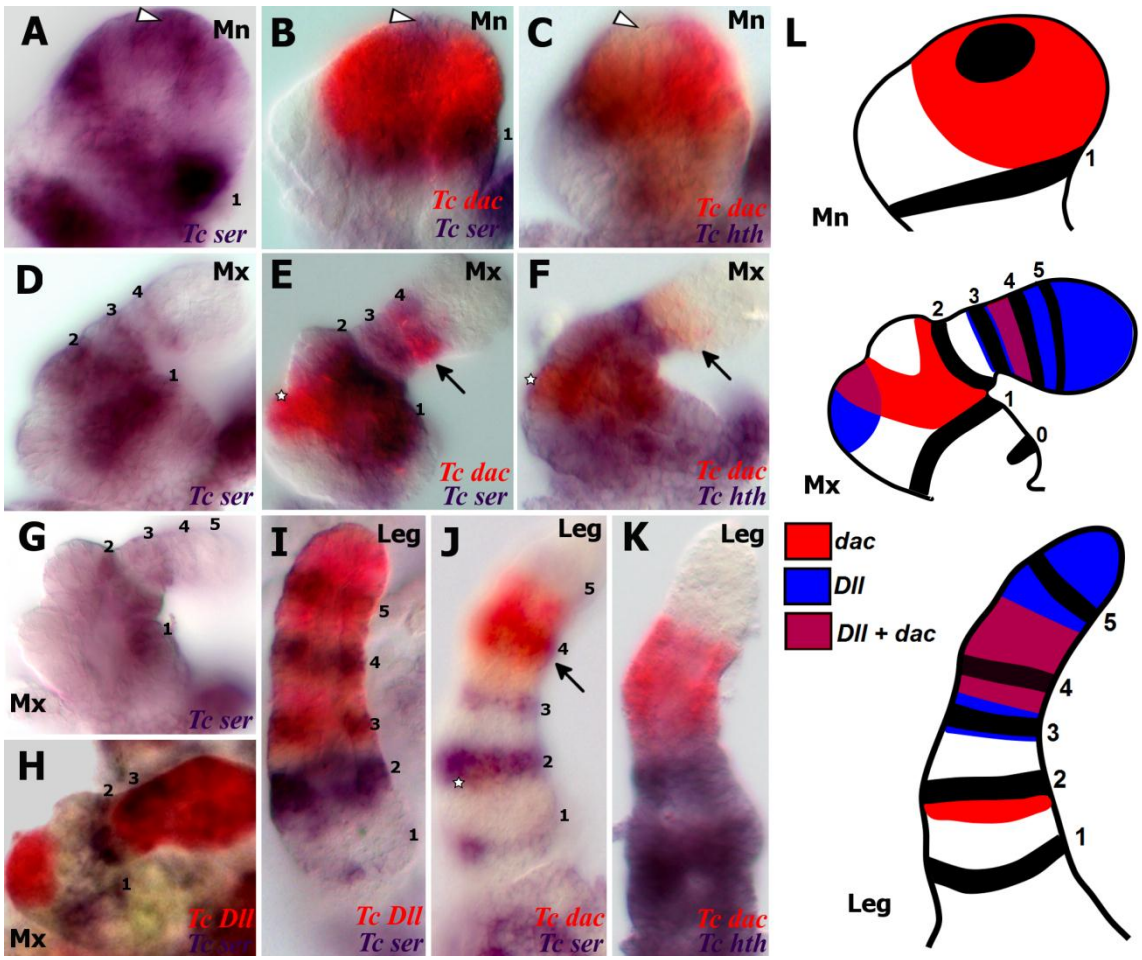

Fig.3.5. Expression of *Tc ser* and the PD domain genes in the developing mandible, maxilla and leg appendages. Gene expression was detected by *in situ* hybridisation. All views are distal to the top and lateral to the right unless otherwise indicated. Expression of *Tc ser* and the genes *Tc Dll, Tc dac,* and *Tc hth* in dissected mandibles (A-C), dissected maxillae (D-H), Dissected legs (I-K). Domains of serrate are numbered 1 to 5 from proximal to distal. (A) lateral view of *Tc ser* expression in the mandible. There is a spot domain in the outer lobe (white arrowhead) and a subcoxa domain that is expressed proximal to the subcoxal groove visible in SEMs. (B) Expression of *Tc ser* (blue) and *Tc dac* (red) in a dissected mandible. *Tc dac* expression is strongest in the outer lobe and coxa of the mandible. (C) Expression ot *Tc hth* (blue) and *Tc dac* (red) in a dissected mandible. *Tc hth* is expression is faint in the inner lobe and stronger at the distal part of the inner lobe. *Tc dac* is expressed strongly in the outer lobe. There is faint expression in the distal part of the outer lobe in to which it appears the spot domain of *Tc ser* fits neatly (Arrowhead). (D) Expression of *Tc ser* in a dissected maxilla. (E) Expression of *Tc ser* (blue) and *Tc dac* (red) in a dissected maxilla. There are two domains of *Tc dac:* a proximal domain (star) and distal (arrow) domain. (F) Expression of *Tc hth* (blue) and *Tc dac* (red) in a dissected maxilla. (G) Expression of *Tc ser* in a dissected maxilla where there is a fifth *Tc ser* domain-5 visible in the maxillary palp. (H) Expression of *Tc ser* (blue) and *Tc Dll* (red) in a dissected maxilla. (I) Expression of *Tc ser* (blue) and *Tc Dll* (red) in a dissected leg. (J) Expression of *Tc ser* (blue) and *Tc dac* (red) in a dissected leg. (K) Expression ot *Tc hth* (blue) and *Tc dac* (red) in a dissected leg. (L) Schematic of *Tc Dll* (blue) and *Tc dac* (red) expression relative to *Tc ser* expression (numbered black stripes).

The cardo/stipes boundary in the larva is shown in fig.3.7G,H and also in a very late embryo shown in fig.2.4D from the previous chapter.

The expression of *Tc ser* is particularly faint and hard to interpret in the labial appendages. *Tc ser* appears to be expressed in the labial appendages in a manner similar to that in the maxilla, with a subcoxal-1 and coxal -2 domain (fig.3.4B,D and fig.3.3A). Labial appendages fuse during late embryogenesis.

There are two rings of *Tc ser* expression in the antenna (see fig.3.3A and fig.3.6). *Tribolium* first instar larvae have an antenna that is made of four segments in proximal to distal order, the antennifer, scapus, pedicellus and flagellum (Toegel et al., 2009). The two rings of *Tc ser* expression to appear first demarcate three segments. The proximal ring is expressed in the distal part of the developing antennifer. The distal ring is expressed in the distal part of the developing scapus. It appears based on the morphology of the appendage that the flagellum develops after the other segments have formed. Expression of *Tc ser* has more complex expression in the antenna distal of the second *Tc ser* domain (fig.3.6B,C), the significance of which is not understood.

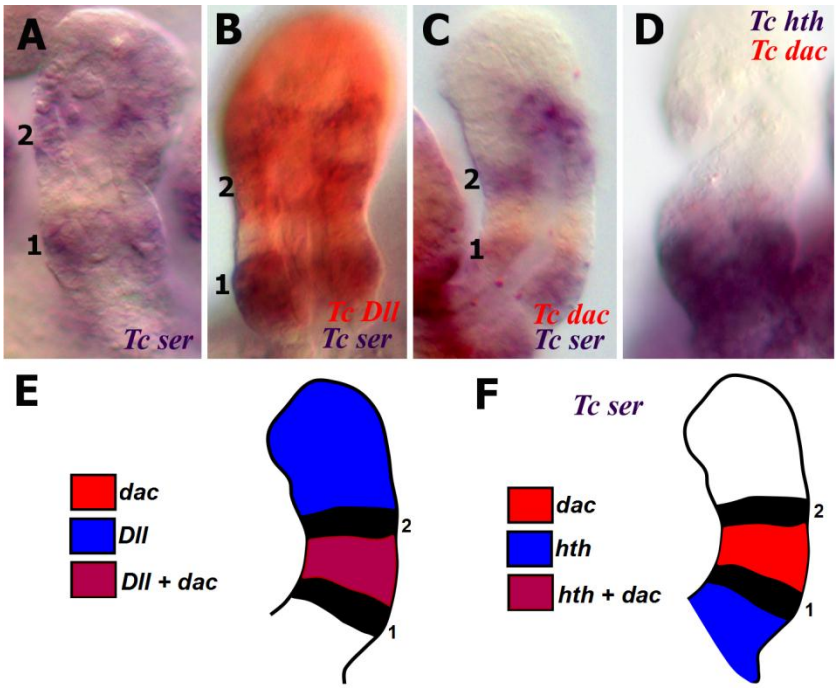

Fig.3.6. Expression of *Tc ser* and the PD domain genes in developing antennae. Gene expression was detected by *in situ* hybridisation. (A) Expression of *Tc ser* in a dissected antenna. (B) Expression of *Tc ser* (blue) and *Tc Dll* (red) in a dissected antenna. (C) Expression of *Tc ser* (blue) and *Tc dac* (red) in a dissected antenna. (D) Expression ot *Tc hth* (blue) and *Tc dac* (red) in a dissected antenna. These expression domains are illustrated E,F (E) Schematic of *Tc dac* and *Tc Dll* expression (F) Schematic of *Tc dac* and *Tc hth* expression in the antenna. *Tc ser* domains are numbered 1 and 3. There are addition *Tc ser* expression patterns distal to the second ring domain that appear after the second ring domain has formed. The significance of this expression is unknown.

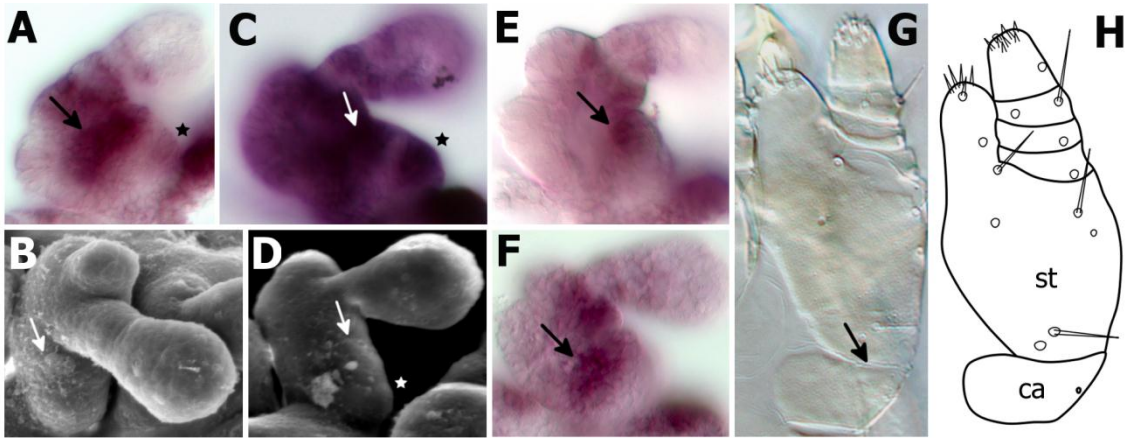

Fig.3.7. Two proximal domains of *Tc ser* expression in the developing maxilla could relate to the future segment boundary between the cardo and stipes. It is not obvious where the cardo/stipes segment boundary develops in the embryonic maxillary appendage. The subcoxal-1 domain of *Tc ser* is clearly associated with a segment boundary. The cardo of the first instar larva is small and the segment boundary with the stipes is close to the base of the maxilla appendage. The appearance of a proximal-0 stripe suggests that this may form the boundary between the cardo and stipes. If this is the case, then the significance of the subcoxal-1 domain of *Tc ser* is unknown. All views are proximal to the top and lateral to the right. Gene expression was detected by *in situ* hybridisation. (A) *Tc ser* expression in a germ band retracted stage embryo. *Tc ser* is strongly expressed in the subcoxal-1 domain (arrow) and very faintly expressed proximal-0 stripe domain (star) more proximal to the subcoxal-1 domain. (B) SEM of a maxilla at a similar stage to that of A. The subcoxal/coxal boundary is indicated with an arrow. (C) Later stage maxilla from an embryo undergoing dorsal closure. The subcoxal-1 domain of *Tc ser* is strongly expressed (arrow). The proximal-0 stipe domain (star) is more strongly expressed than earlier stages. (D) SEM of a maxilla of a similar stage to C. The subcoxal/coxal segment boundary is visible and marked by an arrow. A smaller groove (star) is also visible and is in a corresponding position to the proximal stipe visible in the maxilla in C. (E,F) The subcoxal-1 domain of *Tc ser* is clearly associated with a cell boundary marked by an arrow. (G) Close up of a cuticle preparation from a first instar larval maxilla. The segment boundary between the cardo and stipes is indicated with an arrow. A diagram of the outline of the maxilla is shown in (H).

Tc ser expression in the mandibular limb bud

There are two domains of *Tc ser* expression in the mandibular limb bud, a ring and spot domain (see fig.3.3C and fig.3.5A-C). The spot domain is present on the distal tip of the mandible limb bud, more specifically on the outer lobe of the developing endite that relates to the developing incisor process. *Tc dac* expression appears to be fainter in a similar region to the location of the spot domain (see arrowhead in fig.3.5C). The ring domain of *Tc ser* is situated proximal to the endite (see fig.3.4B-D), and lies proximal to the lateral furrow visible on the scanning electron micrographs of *Tribolium* embryos (compare fig.3.3C with fig.3.3D).

On the lateral part of the mandible, a groove can be seen below the incisor lobe (fig.3.2). The significance of this groove is not easy to determine as in the first instar larva there is no evidence of segmentation or of any groove present on the lateral sheath of the mandible (cuticle on the lateral side of the mandible) that corresponds to

that position (see fig.2.4H,I from chapter two). In consideration of the expression of *Tc ser* expression in the developing mandibular limb bud, this structure is interpreted to mark the division of the mandible into subcoxa and coxa. The evidence in support of this interpretation is detailed below in later sections of this chapter.

Tc prd expression relative to *Tc ser* expression

Tc prd is expressed in the developing endites in the mandibular, maxillary and labial segments (see fig.3.3A). There is also expression of *Tc prd* in the developing mesoderm of the mandible, maxillary, labial and leg appendages (fig.3.3A). This expression is probably located to the developing muscles. *Tc prd* is expressed in the coxa segment, the distal segment of the protopodite. Expression of *Tc prd* is more distal to the subcoxal-1 *Tc ser* domain of the mandible, maxilla and labial protopodite (see fig.3.4B-D).

PD domain gene expression relative to *Tc ser* expression

In order to determine the identity of the ring domains of *Tc ser* expression in all post-antennal appendages, double *in situ* hybridizations of *Tc ser* with *Tc dac* and *Tc Dll* were performed (see fig.3.5, fig.3.8, fig.3.9 and fig.3.10).

In the leg, *Dll* expression clearly overlaps the third, fourth and fifth domains of *Tc ser* (fig.3.5I, fig.3.10A,E,F). These relate specifically to the trochanteral-3, femoral-4 and tibial-5 domains of *Tc ser*. *Tc Dll* is expressed in two domains, a ring and sock domain (seen clearly in fig.2.3D in chapter two). The ring domain overlaps with the trochanteral-3 *Tc ser* domain, the sock domain is co-expressed with the tibial-5 domain (see fig. 35I and fig.3.10A,E,F).

Tc dac has two domains of expression, a proximal domain in the protopodite and a distal domain in the medial region of the telopodite (see fig.3.5J and fig.3.9J). The distal domain of *Tc dac* is coexpressed with the femoral-4 *Tc ser* domain. The proximal domain of *Tc dac* is co-expressed with the coxal-2 *Tc ser* domain. There is a spot of *Tc dac* expression that is present with the subcoxal-1 *Tc ser* domain.

Comparison of *Tc hth* to *Tc dac* expression shows that *Tc hth* is expressed in the subcoxal-1, coxal-2 and trochanteral-3 *Tc ser* domains of the developing leg (fig.3.5K). *Tc hth* expression abuts *Tc dac* expression in the leg. There is no apparent overlap of expression of *Tc hth* and *Tc dac*. The distal domain of *Tc dac* expression relates to the femoral-4 ring of *Tc ser* expression in the leg and is expressed in the developing femur

and tibia segments (fig.3.5J). The proximal domain of *Tc dac* expression, that relates to the coxal-2 ring of *Tc ser* expression, is visible well within the domain of *Tc hth* expression (which encompasses *Tc ser* ring domains 1-3). This is consistent with *Tc hth* being co-expressed with the trochanteral-3 domain of *Tc ser* expression.

In the maxilla of *Tribolium,* the proximal domain of *Tc dac* is expressed before the distal domain in all post-antennal appendages (see fig.3.8 and fig.3.9). Initially, *Tc dac* is co-expressed with the coxal-2 *Tc ser* domain (fig.3.9A). Later in development, the expression of *Tc dac* shifts proximally towards the subcoxal-1 *Tc ser* domain (see fig.3.9D,F,I). *Tc dac* is expressed in both endites, though significantly more strongly in the proximal endite, the lacinia, and expression forms a ring around the coxal-2 segment of the maxilla. There appears to be overlap of the proximal domain of *Tc dac* expression with the first subcoxa domain of *Tc ser* on the lateral side of the maxilla (see fig.3.9I and fig.3.5E). However, this could be an artefact of the manner in which the dissected maxilla was mounted, and reveals the limitations of bright field microscopy. If for example different regions of the maxilla, the subcoxa and coxa, overlap through the line of sight through the microscope, then mutually exclusive expression domains will appear to be co-expressed.

Tc hth can be related to *Tc ser* expression in the maxilla by comparison with *Tc dac* expression. *Tc hth* is expressed throughout the developing protopodite of the maxilla and extends into the proximal region of the palp abutting *Tc dac* expression (see fig.3.5F). Relating *Tc hth* expression to the *Tc ser* ring domains by consideration of the *Tc dac* distal domain in the maxillary palp (fig.3.5E) shows that *Tc hth* is expressed up to the trochanteral-3 domain of *Tc ser* expression. This is reminiscent of the expression of *Tc hth* and *Tc dac* in the leg appendage. *Tc hth* is therefore co-expressed with the third *Tc ser* domain in the maxilla, similar to the leg (compare fig.3.5F with fig.3.5K).

Tc hth is expressed throughout the mandible lobe but is weaker in the inner lobe and the proximal ventral medial region to the molar lobe. *Tc hth* is more strongly expressed in the distal part of the inner lobe (fig.3.5C).

Early expression of *Tc ser* and *Tc dac*

In *Drosophila,* it has been shown that the first *ser* ring to appear is the coxa *ser* domain (Rauskolb, 2001). In order to compare the proximal rings of *Tc ser* in *Tribolium,*

the onset of *Tc ser* expression was compared in different appendage types to the expression to the PD domain gene *Tc dac* (shown in fig.3.8, fig.3.9). The proximal domain of *Tc dac* is upregulated earlier than the distal domain of *Tc dac* (see fig.3.8B) and occurs in the gnathal appendages as soon as the limb buds form (see fig.3.8A).

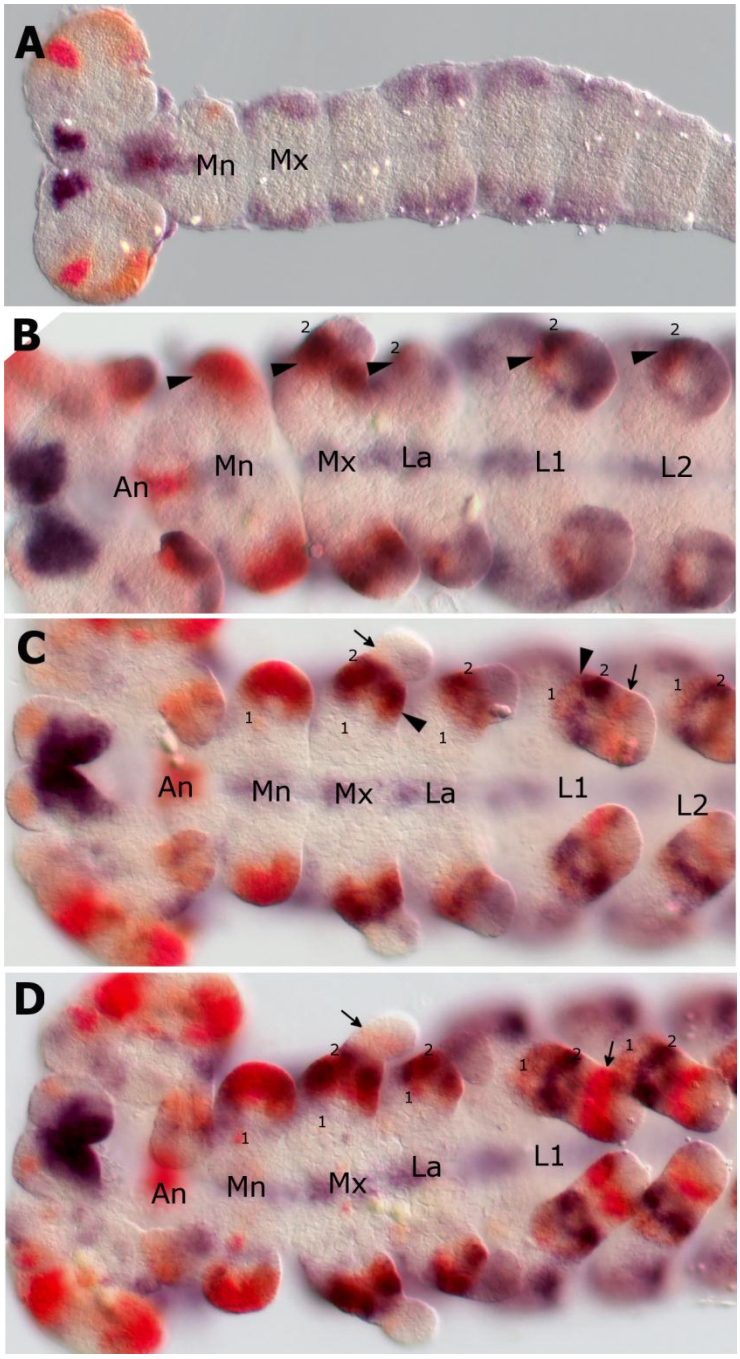

Fig.3.8. Onset of early expression domains of *Tc dac* and *Tc ser* in germ band extending stage embryos. All views are ventral with anterior to the left. *Tc ser* (blue) and *Tc dac* (red) gene expression was detected by *in situ* hybridisation. (A) Germ band extending embryo as the limb buds are forming. Faint *Tc dac* expression is visible in the mandibular and maxillary segments. (B) Germ band extending embryo after the limb buds have formed. The coxal-2 domain of *Tc ser* expression is present in all limb buds except the mandible. The proximal domain of *Tc dac* (arrowhead) is expressed in all post-antennal appendages. (C) The subcoxal-1 domain of *Tc ser* appears more proximal to the coxal-2 domain in all post-antennal appendages. In the legs and maxilla, the distal domain of *Tc dac* (arrow) is expressed along with a distal *Tc ser* ring domain. (D) Fully germ band extended embryo. The distal domain of *Tc dac* is more prominent than earlier stages in the legs and the maxilla (arrow). Mandibular (Mn), maxillary (Mn), labial (La), leg (L1,L2), and antennal (An) segments are indicated.

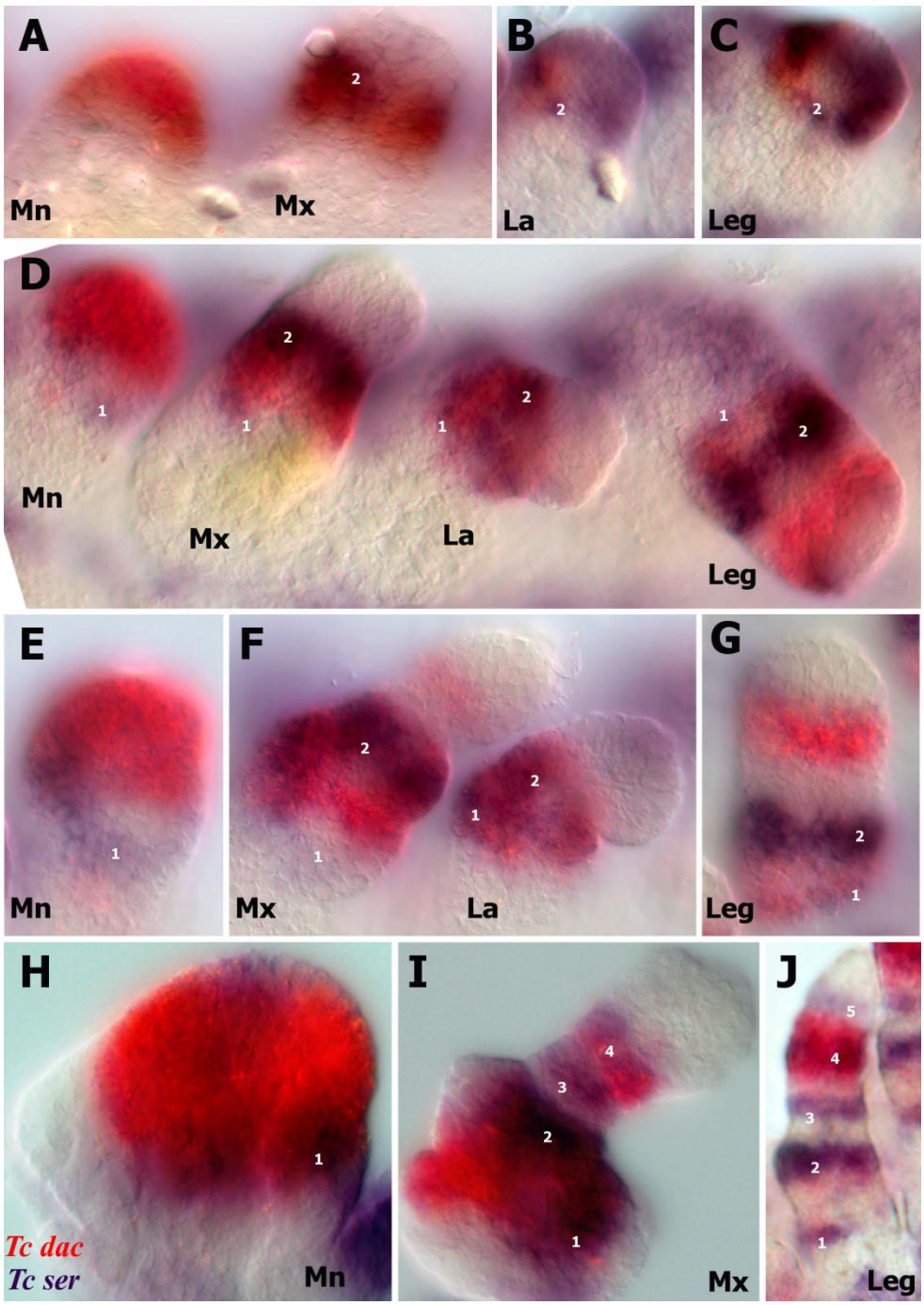

Fig.3.9. Timing of early expression domains of *Tc ser* suggest serial homology of subcoxa and coxa between different appendages. Images show higher magnification of the expression of *Tc ser* (blue) and *Tc dac* (red) from Fig.3.8. Gene expression was detected by *in situ* hybridisation. Figure shows the onset of expression of the first and second *Tc ser* ring domains in the developing limb buds of the mandible, maxilla, labial and leg appendages. The first and second *Tc ser* ring domains are interpreted to represent the distal segment boundaries of the subcoxa and the coxa respectively. Successive germ band extending stage embryos are shown in A-G and germ band retracted stage embryos are shown in H-J. All views are ventral with anterior to the left unless otherwise indicated. (A-C) There are proximal domains of *Tc dac* in each appendage. The maxilla, labial and leg limb buds have a coxal-2 domain of *Tc ser* expression. There is no domain of *Tc ser* in the mandibular segment at this stage. (A) Mandible and maxilla limb buds (B) Labial limb buds. (C) First leg segment. (D) Mandibular, maxillary, labial and first leg segments of a later germ band extending embryo. (E-G) Germ band retracting stage embryos. The proximal domain of *Tc dac* in the leg appendage appears to be broadly expressed between the subcoxal-1 and coxal-2 domains of *Tc ser*. (E) Mandible. (F) Maxilla and labial appendages. (G) First leg appendage. Distal is to the top. (H-J) Germ band retracted stage embryos. Distal is to the top. The majority of *Tc ser* ring domains (1-5) have been activated by this stage. The subcoxal-1 and coxal-2

domains are still present in the leg and maxilla with *Tc dac* expression juxtaposed between the two domains. The mandibular *Tc dac* domain is more distal to the subcoxal-1 *Tc ser* domain. The proximal *Tc dac* domain in the leg is faint by this stage and is adjacent and slightly overlapping with the coxal-2 *Tc ser* domain. In more strongly stained embryos, a proximal spot domain is visible on the subcoxa-1 domain of *Tc ser* (data not shown). (H) mandible. Lateral is to the right. (I) Maxilla. Lateral is to the right. (J) Leg. Ventral view.

Analysis of expression of *Tc ser* and *Tc dac* during early stages of appendage development in *Tribolium* embryos shows the identity and the order in which particular rings of *Tc ser* expression appear. The first ring of *Tc ser* expression to appear in the developing limb appendages is the coxal-2 *Tc ser* ring domain in the maxilla, labial and leg limb buds (see arrowhead in fig.3.8B and fig.3.9A-C). The next proximal domain of *Tc ser* to appear is the subcoxal-1 *Tc ser* ring domain present in the mandibular, maxillary, and leg appendages (see fig.3.8C-D and fig.2.9D-G). Subsequent unidentified telopodite ring domains of *Tc ser* appear (though probably the tibial-5 domain) at a very similar time to the subcoxal domain (see fig.3.9D).

The faintness of the expression of these distal domains as they appear makes conclusions on the precise order of onset of *Tc ser* expression for the entire limb very difficult. For the purposes of this study, I was more interested in the onset of expression of the proximal domains of *Tc ser* that relate specifically to the developing protopodite. For these two domains the results are clear. The coxal-2 ring domain of *Tc ser* appears first in all appendages where it is present (not the mandible) and is co-expressed with *Tc dac* in all segments. The subcoxal-1 domain expressed after the coxal-2 domain and is proximal to the expression of *Tc dac.*

The expression of *Tc dac* is slightly different in the leg, it is associated with the coxal-2 domain throughout embryogenesis (see fig.3.9C,G,J). The proximal *Tc dac* domain in the gnathal appendages are initially co-expressed with the coxal-2 domain (except the mandible) (see fig.3.9A,B). It is then expressed in the coxa segment, migrating proximally towards the subcoxal-1 domain as the endites develop (see fig.3.9D-F, H,I).

The pleuron is derived from the subcoxal segment of the leg

Expression of *Tc ser* reveals the existence of a subcoxal segment present in the developing leg. *Tc ser* expression in late stage embryos shows the developing coxa with a subcoxal-1 domain of *Tc ser* expression more proximal to it close to the thorax, a

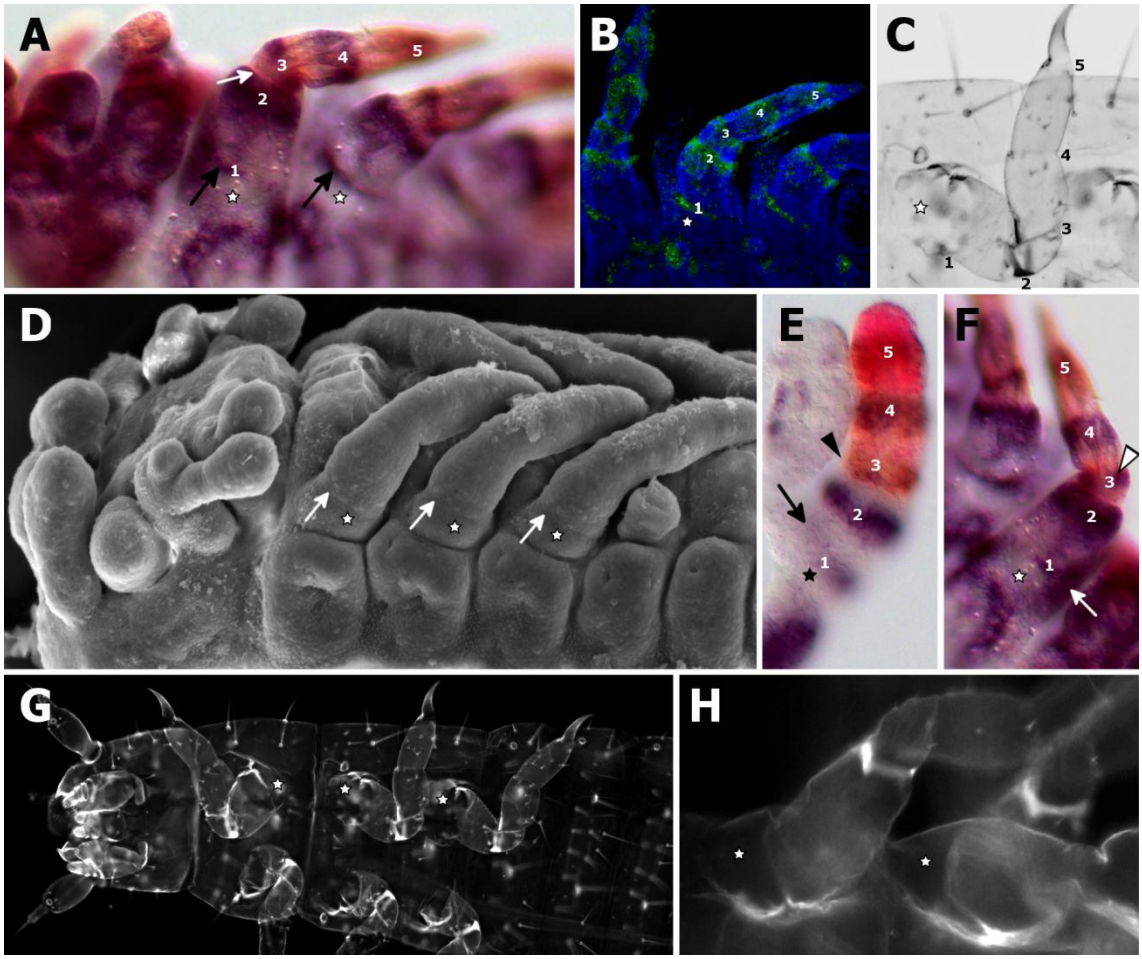

Fig.3.10. The pleuron is subcoxal in origin. By comparing the expression of *Tc ser* and *Tc Dll,* in late stage embryos to both larval legs and earlier embryonic legs reveals that four of the *Tc ser* domains relate to the coxa, trochanter, femur and tibia segments. The proximal *Tc ser* ring domain relates to a subcoxal segment which forms the pleuron in the larva. Anterior is to the left unless otherwise indicated. Arrows mark the position of the subcoxa segment distal boundary. Stars mark the subcoxa segment in the developing embryonic leg and the pleuron in the larvae. Asterisks mark the coxa. The numbers relate to the distal part of the leg segments as follows: 1) subcoxa 2) coxa 3) trochanter 4) femur 5) tibia. Gene expression was detected by *in situ* hybridisation. (A) Expression of *Tc ser* (blue) and *Tc Dll* (red) in a very late stage *Tribolium* embryo undergoing dorsal closure. Lateral view. *Tc ser* is expressed immediately proximal to where the segment boundary forms (white arrow) (B) *Tc ser* expression in a late stage *Tribolium* embryo. (C) Cuticle preparation of a first instar larval leg with corresponding leg segments to A and B indicated. (D) SEM of germ band retracting stage embryo. The subcoxal/coxal segment boundary of the leg is clearly visible as a lateral groove at the base of the leg (arrow). (E) Expression of *Tc ser* (blue) and *Tc Dll* (red) in germ band retracting stage. Distal is to the top and lateral is to the right. The five *Tc ser* ring domains are serially positioned along the P/D axis. The proximal limit of *Tc Dll* expression (arrowhead) is the third ring domain which relates to the trochanter. (F) Expression of *Tc ser* (blue) and *Tc Dll* (red) in a very late stage leg shown in A. The proximal limit of *Tc Dll* expression (arrowhead) is adjacent to the coxa. (G) *Tribolium* first instar larva showing the relative position of the subcoxa (pleuron) to the rest of the larva. (H) Lateral view of the attachment of the larval leg to the body. The pleuron/subcoxa is easily distinguishable as a separated segment.

subcoxal segment (see fig.3.10A,B,F). This segment flattens radially into the body wall and supports the base of the coxal segment of the leg. This leg derived segment is then incorporated into the body wall, although still seen to be separated from it by a segment boundary (see fig.3.10C,D,G,H).

Comparison of the morphology of the larval leg (see fig.3.10C,G-H) to that of the morphology of late stage embryos (see fig.3.10A,F) enables the identity of the *Tc ser* domains of expression to be related to leg segments with confidence. The coxal-2 domain of *Tc ser* expression clearly relates to the coxal segment (white arrow in fig.3.10A). The subcoxal-1 domain of *Tc ser* expression (black arrow in fig.3.10A) and is clearly related to the distal boundary of the developing subcoxal segment of the leg (star in fig.3.10A,B,D-F) that forms part of the pleuron (star in fig.3.10C,G-H).

3.3 Discussion

Study of *Tc ser* expression, as a marker for the position of future appendage segments and *Tc prd* expression as a marker for endite development, shows that the mandible is divided into a coxa and subcoxa. The mandibular endite is attached to the coxa, a more distal segment than the proximal subcoxal segment. This result therefore supports Machida's hypothesis that the embryonic mandible is divided into a subcoxal and coxal segment.

In addition, subcoxal and coxal segments are present in the protopodites of all post-antennal appendages as defined by the expression of *Tc ser* and the position of expression of *Tc prd* and the PD domain genes *Tc hth, Tc dac* and *Tc Dll.* There are significant similarities between the developing subcoxa and coxa of the mandible and the developing maxilla as defined by the expression of these genes. These similarities presumably indicate serial homology of the subcoxa and coxa segments of the mandible and maxilla as hypothesized by Machida. If this is the case, the ancestral protopodite of the gnathal appendages would have likely originally consisted of three segments: a subcoxa, coxa and basis (using crustacean terminology), with the mandibular gnathal edge located on a more distal segment, the coxa, which is not the most proximal segment as Boxshall and others have hypothesized.

There is strong evidence for the existence of a subcoxal leg segment that forms the pleuron in the larva. This result therefore supports the subcoxal theory of the origin of the pleuron. There are some similarities between the subcoxa and coxa of the gnathal appendages to the subcoxa and coxa of the developing legs. The similarities between the gnathal appendages and the legs may indicate possible homology of the leg subcoxa to the subcoxa of the gnathal appendages, although the similarities are less significant than those observed between the mandible and maxillary appendages.

Mandible limb bud is divided into subcoxa and coxa

The most significant result of this chapter was the discovery of the expression of a ring of *Tc ser* in the developing mandible. This expression domain is interpreted to represent the distal segment boundary of a subcoxa. The *Tribolium* mandible is an unsegmented appendage in both the larva and adult like all hexapods. However, a lateral groove is present in the mandibular limb bud in the developing embryo as visualized by SEM and clearly relates to the expression of *Tc ser*. The expression of *Tc*

ser is in a similar position on the proximal distal axis to the groove visible in the maxillary segment (shown in fig.3.2, fig.3.3).

This lateral groove on the mandible has been detected in other hexapods. Machida hypothesized that a lateral groove present in the jumping bristletail *Pedetontus unimaculatus* was evidence of vestigial segment boundary and was serially homologous to the cardo/stipes segment boundary in the maxilla (Machida, 2000). The lateral groove is present in both the cricket *Gryllus* (Liu et al., 2010) and the sawfly *Athalia rosae* (Oka et al., 2010). The authors of the study of *Athalia* define the segment more proximal to this groove as the subcoxa segment. However, there was no genetic evidence for the subdivision of the mandible into subcoxa and coxa.

The ring domain of expression in the mandible could be evidence of an ancestral segment boundary, or some other functional developmental boundary that doesn't reveal itself through the formation of an actual segment joint. Notch signalling is often involved in demarcating different populations of cells (Bray, 2006). The ring of *Tc ser* expression could represent some particular boundary of cells, such as those present in the developing endites as the subcoxal ring domain is expressed proximal to all developing endites. This interpretation seems unlikely given the similarities of the subcoxa domain of expression to other segment forming domains. The similarites of the proximal *Tc ser* domain in the mandible to the maxilla, labial and leg appendages instead suggests that the mandible subcoxa is serially homologous to the subcoxa present in other appendages.

No apparent expression of *Tc ser* in the gnathal appendage endites

Sewell *et al.* suggested from evidence in *Triops* that *notch* signalling is associated with endites[11] (Sewell et al., 2008). There is no evidence of any endite specific *Tc ser* domains in the mandible, maxilla or labial appendages in *Tribolium,* and therefore the Notch signalling pathway does not appear to have a role in endite development, at least in *Tribolium.*

[11] Although, there was no description of the observed expression pattern of any members of the Notch signalling pathway, so it is difficult to make a fair comparison.

Segmental identity of the *Tc ser* expression domains in the leg.

By comparing the expression of the PD domain genes to *Tc ser* expression, and comparing the expression of *Tc ser* to the morphology of the developing legs in late stage embryos it is possible to define the segmental identity of each *Tc ser* domain. The largest segment of the larval leg, and the most recognizable, is the coxa. It forms a cone-like shape at the base of the leg. The characteristic morphology of the coxal segment is recognizable in late embryogenesis, as is the morphology of the other segments (compare fig.3.10A-B,F to fig 3.10C,G-H). There is clearly a coxal-2 *Tc ser* domain associated with the distal part of the coxa next to the coxal-trochanter boundary (white arrow in fig.3.10A).

In the developing leg there are five domains of *Tc ser* expression (see fig.3.10E). These five domains of *Tc ser* relate to the five segment boundaries in the larval leg (fig.3.10C). The coxal-2 *Tc ser* domain is the second ring domain of *Tc ser* and clearly relates to the distal part of the coxal leg segment. This is confirmed by the expression of *Tc Dll* in the third *Tc ser* domain in an earlier developing leg appendage (arrowhead in fig.3.10E) which is co-expressed with the trochanteral-3 domain that relates to the trochanter in a later stage developing leg appendage (arrowhead in fig.3.10F). The trochanter is recognizable as a one of the smallest leg segments adjacent to the coxa, the largest leg segment.

By comparing the expression of all the PD domain genes to *Tc ser* expression, the following conclusions can be made: *Tc Dll* is co-expressed with the trochanteral-3, femoral-4 and tibial-5 domains of *Tc ser.* The distal domain of *Tc dac* is co-expressed with the femoral-4 domain of *Tc ser*. The proximal domain of *Tc dac* expression is located in the coxa segment. *Tc hth* is co-expressed with the subcoxal-1, coxal-2 and trochanteral-3 domains of *Tc ser*. This is shown schematically in fig. 3.11.

The proximal domain of *Tc dac* expression is expressed in the coxal segment.

Prpic *et al.* argued that the proximal domain of *Tc dac* supported serial homology of the whole mandible to the coxal leg segment (Prpic et al., 2001). The expression of *Tc dac* relative to *Tc ser* indicates that the proximal domain of *Tc dac* is expressed in the coxa of the developing leg: the proximal domain of *Tc dac* is co-expressed with the coxal-2 domain of *Tc ser*. The proximal domain of *Tc dac* in other appendages is also present in the coxal segment. These results therefore support the

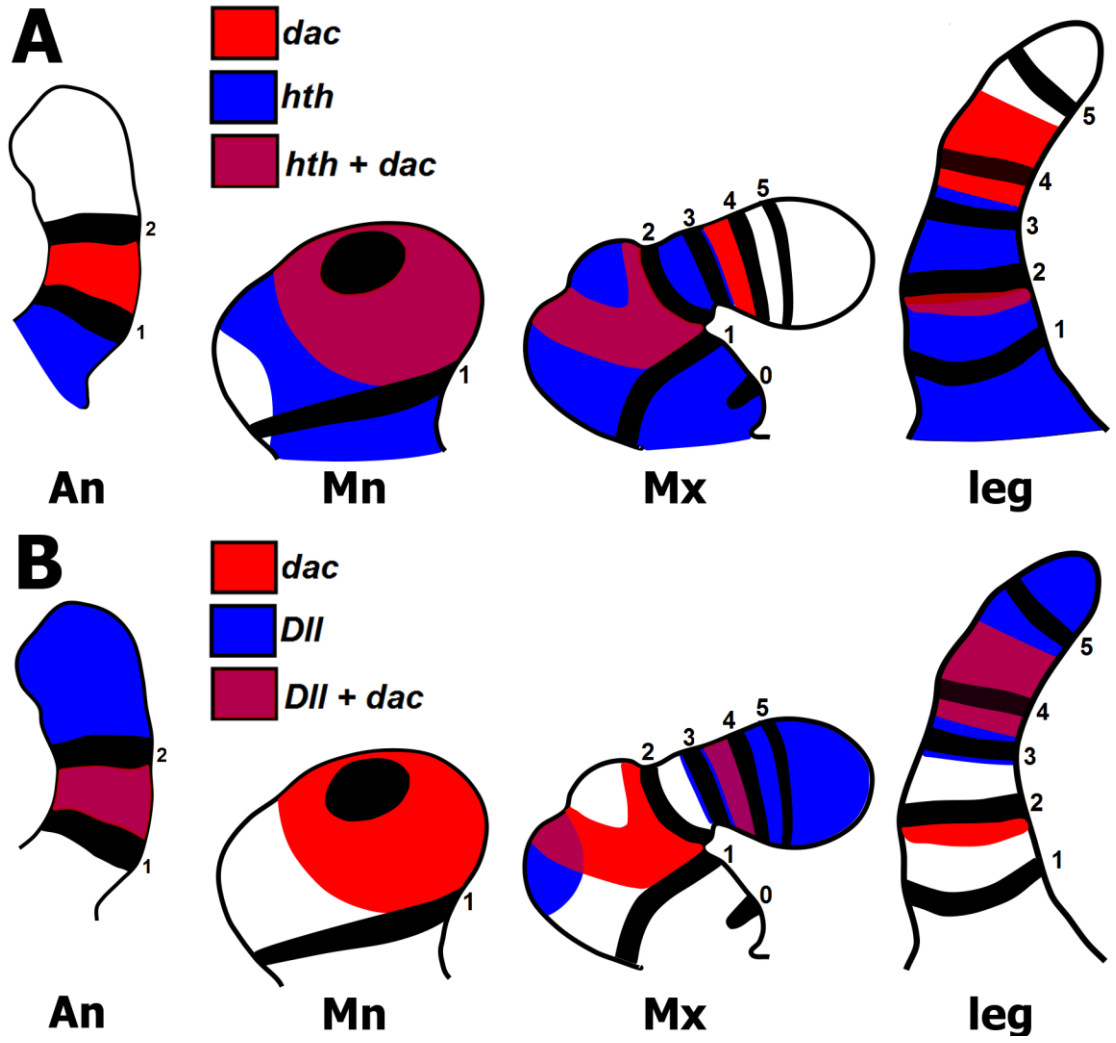

Fig.3.11. Similarity of expression domains of *Tc ser* relative to the PD domain genes in different post-antennal appendages suggests serial homology. All views are distal to the top. The mandible and maxilla are orientated with lateral to the right. *Tc ser* ring domains are marked as numbered black lines. Based upon the similarity of the expression of the PD domain genes to particular domains of *Tc ser* expression in each appendage, each *Tc ser* domain is numbered in order from proximal to distal. *Tc ser* is expressed in the distal part of each segment adjacent to where the segment boundary will form. In the leg, 1 refers to the expression of *Tc ser* in the distal boundary of the subcoxa, 2 to the coxa, 3 to the trochanter, 4 to the femur, and 5 to the tibia. In the mandible, maxilla and labial appendages, 1 and 2 refer to the distal boundaries of the subcoxa and coxa respectively. The proximal-0 stripe domain of *Tc ser* in the maxilla is different to subcoxal ring domains present in other appendages and has therefore been numbered 0. (A) Representation of the expression domains of *Tc dac* (red) and *Tc hth* (blue). Overlapping regions are purple. *Tc ser* ring domains are marked as numbered black lines. (B) Representation of the expression domains of *Tc dac* (red) and *Tc Dll* (blue). Overlapping regions are purple.

conclusions of Prpic *et al.* that the proximal domain of *Tc dac* is expressed in the coxa and is evidence for the serial homology of these appendages. There are, however, differences between the proximal domain of *Tc dac* expression in the gnathal appendages compared to the legs. More proximally to the proximal domain of *Tc dac,* it is apparent that there is a domain of *Tc ser* in all post-antennal appendages. This is

the subcoxal-1 domain of *Tc ser* expression, and provides evidence that a subcoxal segment is present in all post-antennal appendages.

The subcoxa of the leg becomes the pleuron

The subcoxal-1 domain of *Tc ser* expression domain provides molecular evidence for the subcoxal derivation of the pleuron in *Tribolium* larvae and therefore supports the subcoxal theory of the origin of the pleuron, at least for insects. The pleuron is defined as the part of the body where the legs attach to the thorax (Snodgrass, 1935; Boxshall, 2004; Grimaldi and Engel, 2005). Expression of *Tc ser* reveals the existence of a subcoxal segment present in the developing leg. *Tc ser* expression in late stage embryos shows the developing coxa with a subcoxal-1 domain of *Tc ser* expression more proximal to it close to the thorax, a subcoxal segment (see fig.3.10A,B,F). This segment flattens radially into the body wall and supports the base of the coxal segment of the leg. This leg derived segment is then incorporated into the body wall, although still seen to be separated from it by a segment boundary (star in fig.3.10C,D,G,H).

In the majority of insects, the coxa is attached to separate sclerites which are hypothesized to have subcoxal origin (Snodgrass, 1935; Boxshall, 2004). In the *Tribolium* first instar larva, the leg subcoxa does not differentiate into separate sclerites and is visible as a separate segment. The legs of *Tribolium* larvae are attached to a flattened subcoxal segment on thoracic segments. This condition is found in numerous larval insects (Snodgrass, 1935).

The proximal-most segment has been defined in the leg of the sawfly *Athalia* as a subcoxa (Oka et al., 2010). However, to date there has been no molecular evidence for the subcoxal derivation of the pleuron.

Some authors have considered the pleural sclerites to be a homologous character that unites myriapods and hexapods in the clade Tracheata (Bäcker et al., 2008). This explanation is unlikely, due to the high degree of support for monophyletic Pancrustacea. The presence of sclerites that are derived from appendage segments is more likely to be evidence of convergent evolution, possibly as an adaptation to terrestial locomotion.

Homology of the subcoxa and coxa of the mandible to the subcoxa and coxa of other post-antennal appendages

Comparison of the subcoxa and coxa of the mandible reveals striking similarities in the expression of PD domain genes relative to other subcoxal and coxal segments. The subcoxa and coxa of the post-antennal appendages show numerous similarities of gene expression that suggest serial homology. This conclusion is based upon comparisons of *Tc ser* expression which define segment boundaries to the expression of the PD domain genes which define segment identities, especially that of *Tc dac*. The similarities are particularly notable between the mandible and maxilla subcoxa.

The coxal segment of the gnathal appendages is characterized by the expression of *Tc prd* in the developing endites (see fig.3.4) and the proximal domain of *Tc dac* (see fig.3.5). The subcoxal segment is characterized by exclusive expression of *Tc hth* (see fig.3.5).

Additional evidence for the serial homology of the coxa and subcoxa is the sequence of activation of the subcoxal-1 and coxal -2 *Tc ser* domains in the developing limb appendages. The first *Tc ser* domain that is activated is the coxal-2 ring domain. The coxa domain is activated first in all post-antennal appendages except the mandible and is co-expressed with *Tc dac* (shown in fig.3.12A). The subcoxal-1 ring domain is activated next in all post-antennal appendages, including the mandible, proximal to the proximal domain of *Tc dac* (shown in fig.3.12B). The first ring of *Tc ser* expression to appear is the coxal ring of expression. The second ring of *Tc ser* expression to appear is the more proximal subcoxal *Tc ser* ring. The appearance of both of these rings so early in the development of the limb bud in all appendages suggests that these *Tc ser* domains are fundamental segment divisions of the developing appendages. The simultaneous activation of the subcoxal-1 domain of *Tc ser* suggests that the two domains share the same developmental pathway which may indicate serial homology between the gnathal appendages.

Machida hypothesized that a lateral groove present in the mandible of the jumping bristletail *Pedetontus unimaculatus* was serially homologous to the cardo-stipes segment boundary in the maxilla (Machida, 2000). The lateral groove present in *Gryllus, Athalia* and *Tribolium* is in a similar position on the mandibular and maxillary

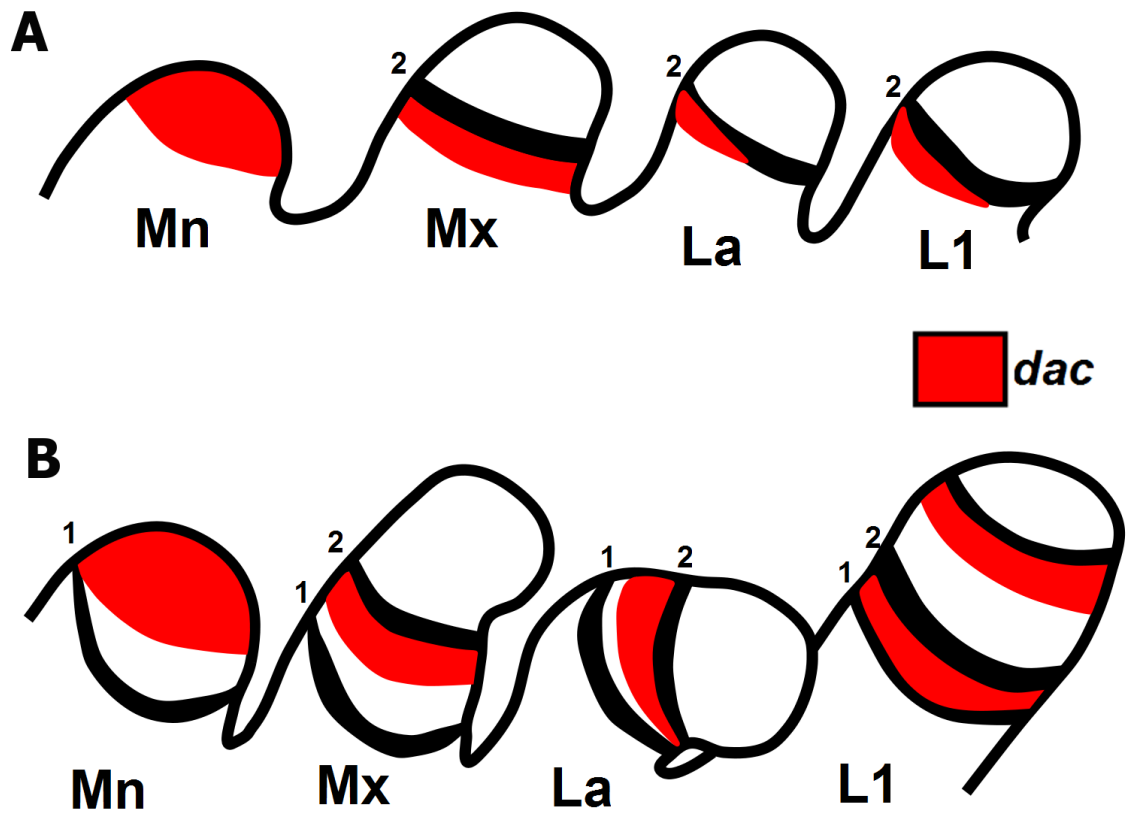

Fig.3.12. Expression of *Tc ser* and the proximal *Tc dac* domain suggests serial homology of the coxa and subcoxa. The schematic of this figure is adapted from the *in situ* hybridisations shown in figure 3.9. The figure shows the onset of expression of the first and second *Tc ser* ring domains in the developing limb buds of the mandible, maxilla, labial and leg appendages. (A) Early limb bud formation. Expression of the coxal-2 domain of *Tc ser* is associated with the expression of the proximal domain of *Tc dac*. This occurs at the same time in all post-antennal limbs. (B) Subsequent expression of the subcoxal-1 domain of *Tc ser* in later developing limb buds. The distal domain of *Tc dac* is present in the leg appendages.

appendages and seems to represent a shared ontogenetic program between the mandibles and maxillae of these species.

The simplest explanation for the identity of the subcoxal ring domain of *Tc ser* in the mandible and maxilla is that the similarity of expression reflects their serial homology. Homology of the mandible subcoxa to the maxilla subcoxa depends on common ancestry of those structures in question and not just their similarity of structure or gene expression patterns although homology will often reveal itself through similarity of structure or patterning mechanism. Similarity could represent convergent evolution. Therefore care has to be taken in order not to mistake convergent evolution as homology. The diversity of arthropod appendages and the relationship of these appendages on a phylogenetic tree must be taken into account before similarities between characters can be understood as homologies or homoplasies.

Arthropod post-antennal appendages evolved from serially homologous biramous limbs. At some point in the evolution of the mandible and maxilla, the limbs were identical in structure and then diverged to evolve into the myriad forms of mandibles and maxillae that are present today. The implications of a serial homologous relationship between the subcoxa of the mandible and maxilla are that the ancestral protopodite of the gnathal appendages would have originally consisted of three segments: a subcoxa, coxa and basis (using crustacean morphological terminology). The mandibular endite is present on the mandibular coxal segment, which is more distal than the subcoxal segment. The mandibular endite could be homologous to one of the endites present on the maxillary coxal segment, the lacinia. The segmented diplopod mandible consisting of a cardo (subcoxa) and stipes (coxa) could represent the primitive state. The basis has been lost in the insect mandible.

In the insect maxilla, it is hypothesized that maxillary stipes segment has formed from fusion of the coxa and basis which is present in more primitive hexapod maxillae (Boxshall, 2004). This fusion of the coxa and basis to form the stipes of the insect maxilla does complicate proposed homologies between the coxa of the mandible to the coxa (or stipes) of the maxilla which have been suggested in this chapter. The proposed solution to these difficult questions of serial homology of arthropod appendage segments is to acquire more data (particularly of Notch signalling pathway and PD domain gene expression) from more taxa.

The implications of the serial homology of the subcoxa between appendages will be investigated in chapter seven in more detail.

Serial homology of the gnathal appendage subcoxa to the subcoxa of the leg

Boxshall has commented that if the pleuron of the leg has a subcoxal origin then it is possible to serially homologize the leg protopodite segments to other appendages (Boxshall, 2004). The expression of *Tc ser* indicates that there is a subcoxal segment in the leg that develops into the pleuron in the larva confirming the subcoxa hypothesis of the origin of the pleuron. There are numerous similarities between the developing subcoxa and coxa of the leg to the subcoxa and coxa of the gnathal appendages that suggest serial homology.

The similarity of the expression of the PD domain genes relative to *Tc ser* domains in the subcoxa and coxa post-antennal appendages (fig.3.11) provides some

evidence of homology. In addition, the coxal-2 domain of *Tc ser* expression is activated at the same time in the maxilla, labial and leg limb buds (fig.3.12). The proximal domain of *Tc dac* is activated with the coxal-2 domain of *Tc ser.* The subcoxal-1 domain of *Tc ser* is activated at the same time in all post-antennal appendages (shown in fig.3.12).

Accepting all these similarities of gene expression as evidence of serial homology, the leg coxa would be serially homologous to the maxillary stipes, the mandibular coxa and to the labial prementum. The leg subcoxa would be serially homologous to the maxillary cardo, the mandibular subcoxa and the labial postmentum (fig.7.1 and fig.7.2).

However, there are some subtle differences between the proximal domains of *Tc dac* of the leg appendages compared to the gnathal appendages. These differences may reflect fundamentally different patterning mechanisms of these coxal segments, or may result from the different morphology of the limbs based on the presence of endites in the gnathal appendages.

There are in fact three domains of expression of *Tc dac* in the developing leg (Prpic et al., 2001), a distal domain in the femur, a larger proximal domain which overlaps with the coxa domain of *Tc ser,* and another smaller spot of expression which is co-expressed with the subcoxa domain of *Tc ser* (data not shown). The proximal domain of *Tc dac* expression in the leg is co-expressed with the coxa domain of *ser* expression. This is in contrast to *Tc dac* expression in the gnathal appendages where the proximal expression domain is expressed in between the coxa and subcoxa domains of *Tc ser* expression domains in the developing coxa.

Does the *Tc ser* subcoxal ring domain of the maxilla correlate to the cardo segment boundary?

The evidence for the homology between the mandible subcoxa and coxa to the maxilla subcoxa and coxa has been presented above. One question that remains to be answered is whether the developing cardo-stipes segment boundary is the subcoxal-1 domain of *Tc ser* expression.

As I had trouble to locate unambiguously the precise position of the developing cardo-stipes segment boundary, the subcoxal-1 domain of the maxilla may not relate at all to the position of the cardo. Instead the proximal-most domain-0 of *Tc ser* may

represent the future cardo segment (see fig.3.5L and star in fig.3.7A,C). This domain is significantly different to the subcoxal-1 domain in terms of timing of expression and relative expression of the PD domain genes and *Tc prd* as an endite marker.

The subcoxal-1 domain may be involved in patterning some aspect of the proximal gnathal appendages, for example, by providing positional information to pattern the endites. The gnathal appendage endites are all present in the coxa immediately distal to the subcoxal-1 domain of *Tc ser* (see fig.3.4D).

It was not possible to perform *in situ* hybridizations with *Tc ser*, or to obtain SEM images of the developing maxilla on very late stage embryos, later than those shown in the figures above. These might have confirmed the position of the division of the maxilla protopodite into cardo and stipes segments. As a result, there is uncertainty as to the position of the cardo-stipes boundary on the developing maxilla. It is plausible that the subcoxa ring of *Tc ser* expression relates to the future cardo-stipes boundary, and certainly marks the position of some sort of boundary in the developing maxilla (see fig.3.7E,F).

It seems just as reasonable to hypothesize that the proximal stripe of *Tc ser* (proximal-0 *Tc ser* domain) that appears later in maxilla development than the subcoxa ring domain could relate to the position of the future cardo and stipes division. In which case, the subcoxal-1 *Tc ser* domain in the maxilla would relate to some other aspect of development than the development of the cardo/stipes segment boundary. The subcoxal-1 *Tc ser* domain could reflect the evolutionary history of the maxillary appendage as a vestigial segmental expression domain, or it could be involved in some other aspect of appendage development, such as the development of the endites.

General conclusions

In order to determine whether the subcoxa and coxa are serially homologous between the mandible and maxilla, and also to the labial and leg appendages, it is necessary to compare the expression of the PD domain genes relative to the Notch signalling pathway in other arthropod taxa. Evidence from one taxon, *Tribolium,* of similar expression patterns in different appendages is not significant enough to provide strong support for particular serial homology hypotheses. By relating the expression of *ser* to particular leg segments and to PD domain gene expression it is possible to define the precise segmental expression the PD domain genes. With this knowledge it will be

much easier to compare segments between different appendages and between diverse taxa without requiring functional genetics (which is a major constraint in evolutionary developmental studies). Study of the expression of PD domain genes and the Notch signalling pathway in biramous limbs could also be informative, by relating the molecular development of the PD axis to the morphological definitions of the protopodite (defined as where the two branches of the biramous limb, the telopodite and exopodite, attach). In particular, the coxal-2 domain of *Tc ser* expression, considering its early activation in all appendages with telopodites and its proximity to the likely boundary between the protopodite and telopodite may relate to the protopodite/telopodite boundary. If this is the case, then this *ser* domain may be present in the distal segment of the protopodite in other appendage types like biramous limbs.

If relationships between the Notch signalling pathway and the PD domain gene expression reveal further similarities between the subcoxa and coxa of the mandible to the maxilla, it would support serial homology of the subcoxa of the mandible and maxilla, and maybe to the leg appendages. The plausibility of a plesiomorphic three segmented protopodite could then be seriously evaluated.

Chapter 4:

Tc cnc differentiates the mandible from a maxilla in *Tribolium*

4.1 Introduction

In *Drosophila, cap'n'collar* (*cnc*) has been shown to pattern the mandibular segment (Mohler et al., 1995). *cnc* differentiates the mandibular segment from the maxillary segment by repressing the Hox gene *Dfd.* In this chapter, the function of the *Tribolium* homologue of *cnc* was explored by knocking it down by parental RNAi to determine whether it has a conserved role in patterning the mandibular segment of *Tribolium.*

The arthropod mandible is an appendage generally adapted for biting and chewing. All post-antennal arthropod appendages have evolved from a biramous limb (Boxshall, 2004; Waloszek et al., 2007; Chen, 2009). The biting/chewing structure that forms the gnathal edge of the mandible is present on the base (the protopodite) of this ancestral biramous limb.

The mandible has most likely evolved from a biramous maxilla-like precursor by modification of the proximal endite to form the gnathal edge. Such a maxilla like precursor is present in numerous stem lineage arthropod fossils such as *Martinssonia elongata* (Muller and Waloszek, 1986) and Phospatocopida (Siveter et al., 2001; Edgecombe, 2010; Rota-Stabelli et al., 2011). Numerous variations to the structure of the ancestral mandible have occurred, such as loss of the mandibular palps. However the defining characteristic of the mandibular appendage shared by mandibulate arthropods is the presence of this gnathal edge, consisting of a molar process and incisor process, on the protopodite. This gnathal edge is widely considered to be a synapomorphy of the clade Mandibulata (Kraus, 2001; Edgecombe et al., 2003; Edgecombe, 2010).

Arthropod phylogeny and the evolution of the mandible

The mandible is present in two extant arthopod lineages, Pancrustacea (insects and crustaceans) and Myriapoda (millipedes and centipedes). Insects and myriapods were previously grouped together in the Atelocerata (also known as Tracheata). As a result of robust molecular phylogenetic evidence, together with a suite of morphological characters, insects are now grouped with crustaceans to form the Pancrustacea.

The position of the Myriapoda is less clear. Two competing hypotheses are favoured by different molecular phylogenies: placing the myriapods as sister group to the Pancrustacea to form Mandibulata, or placing the myriapods as a sister group to the Chelicerata to form Myriochelata. Morphological evidence strongly favours Mandibulata over Myriochelata. More recent molecular phylogenies that have larger datasets, include evidence from rare genomic changes and take more care over outgroup choice favour Mandibulata (see chapter one for literature reviews).

These two competing phylogenetic hypotheses have different implications regarding the evolution of the mandible. The most commonly suggested path of mandible evolution is of a single origin in the ancestor to the myriapods, crustaceans and insects (Snodgrass, 1938; Edgecombe, 2010). This hypothesis is compatible with the clade Mandibulata, not surprisingly as the name Mandibulata suggests. There is also the formal possibility, considered unlikely, that the mandible has evolved independently more than once within Mandibulata.

If myriapods were grouped with the chelicerates in Myriochelata it would suggest that the mandible had evolved independently in the Myriapoda and Pancrustacea (Mayer and Whitington, 2009). The homologous segment of the chelicerates to the mandibular segment is the first leg segment (L1). The ancestral leg appendage of the Chelicerata on the L1 segment was almost certainly a biramous limb. The ancestor to Myriochelata would have therefore possessed a leg appendage on the mandibular/L1 segment. A single evolutionary origin of the mandible appears extremely unlikely under the Myriochelata hypothesis. As sister group to the mandibulate myriapods, the chelicerate first leg appendage would have had to evolve from a mandible.

If the mandible is a homologous appendage across Mandibulata it would suggest that there may be significant similarities in the embryonic development of the

mandible and the mandibular segment between diverse lineages of this clade. By studying the genes involved in patterning the mandible of *Tribolium,* a mandibulate insect, and by comparing these genes to other mandibulate taxa, it may be possible to demonstrate the homology of the mandible across mandibulates and inform the phylogenetic dispute regarding the placement of the Myriapoda. In addition, the manner in which the mandible is patterned may show evidence for the evolution of the mandible from a maxilla-like precursor.

Mandible segment patterning genes in *Drosophila*

As a preliminary step, candidate mandibular segment patterning genes were chosen from *Drosophila* to be considered for research in *Tribolium.* Research on *Drosophila melanogaster* has revealed several segment patterning genes, *cap'n'collar (cnc), Deformed (Dfd)* and *apontic (apt),* to be required for patterning the mandibular segment. There is however, no known homologue of *apt* in *Tribolium* and therefore effort was concentrated on studying the function of *cnc* and *Dfd*.

Hox genes are master regulatory genes that give segments their individual identity. Hox genes activate downstream targets that define and pattern segments. One Hox gene, *Dfd,* is expressed in the mandibular segment of all mandibulate arthropods. In *Drosophila Dfd* is expressed in the mandibular and maxillary segments during embryogenesis. *Drosophila* that are mutant for *Dfd* are missing maxillary and mandibular derived structures (Merrill et al., 1987; Regulski et al., 1987; McGinnis et al., 1998; Brown et al., 1999; Veraksa et al., 2000).

cnc function in *Drosophila*

Dfd is responsible for patterning both the mandibular and maxillary segments, but does not differentiate the mandibular segment from the maxillary segment. For this function another gene, *cnc,* is involved. *cnc*, a basic Leucine zipper family gene (bZIP), is necessary for the development of labral and mandibular derived structures and achieves this in part by repressing the maxilla patterning function of *Dfd. cnc* is expressed in an anterior 'cap' domain in the labrum and a posterior 'collar' domain in the mandibular segment. *cnc* null mutants lose both labral and mandibular segment derived structures and have a duplication of maxilla derived structures (Mohler et al., 1995; McGinnis et al., 1998; Veraksa et al., 2000).

Hypopharyngeal lobe derived structures are lost in *cnc* null mutants and are ectopically produced with ectopic expression of *cnc* (Veraksa et al., 2000). The hypopharyngeal lobes are often thought to be derived from the intercalary segment (Rogers and Kaufman, 2007; Veraksa et al., 2000), however it has recently been shown by our laboratory that the hypopharyngeal lobes are derived from the mandibular segment in agreement with earlier hypotheses (Economou and Telford, 2009).

cnc genetic interactions

Dfd expression is repressed by *cnc* in the anterior of the mandibular gnathal lobe. The activity of Dfd protein is also repressed by *cnc* in the mandibular segment. In addition to the repression of *Dfd* expression and activity in the mandibular segment of the developing fly embryo, *cnc* represses the expression of the Hox gene *proboscipedia (pb)* (Rusch and Kaufman, 2000). *pb* has no known function in the patterning of the fly embryo as *pb* mutants have no apparent embryonic phenotype (Rusch and Kaufman, 2000). *cnc* also represses the ventral expression domain of the PD domain gene *Dll* (McGinnis et al., 1998; Rusch and Kaufman, 2000). *cnc* itself is activated by gap and pair rule genes and is regulated independently of any Hox genes (Mohler, 1993).

Dfd expression across Mandibulata

Dfd and *cnc* are required to pattern the mandibular segment in *Drosophila.* It appears that based on the conserved expression of *Dfd* and *cnc,* they will have a similar role in mandibulate insects and myriapods. *Dfd* is expressed in the mandible and maxilla bearing segments in the majority of mandibulates, although with some variation (Hughes and Kaufman, 2002a). Insect species that have been investigated include *Drosophila melanogaster* (Diederich et al., 1991) *Tribolium castaneum* (Brown et al., 2000) *Acheta domestica, Thermobia domestica, Oncopeltus fasciatus* (Rogers et al., 2002), *Bombyx mori* (Kokubo et al., 1997) and *Apis mellifera* (Walldorf et al., 2000). Crustacean species that have been studied include the woodlouse *Porcellio scaber* (Abzhanov and Kaufman, 1999a) and the water flea *Daphnia pulens* (Papillon, unpublished). Two myriapods have been studied, a chilopod *Lithobius atkinsoni* (Hughes and Kaufman, 2002b) and a diplopod *Glomeris marginata* (Janssen and Damen, 2006).

There are some differences between the different groups, but in general the expression patterns are conserved with expression limited to the mandibular and

maxillary segments. Expression in *Lithobius* also includes the 2nd maxillary segment which appears to indicate that *Dfd* is involved with patterning the mandibles and maxillae of this species (Hughes and Kaufman, 2002b). Expression data from crustaceans indicates that the expression domain is more restricted to the mandibular segment.

After early expression in the mandibular and maxillary segments, there is dynamic expression of *Dfd* in the mandibular segment in mandibulates in a manner that resembles the dynamics of *Dfd* expression in *Drosophila.* The region from which *Dfd* expression retracts is called the distal part or the central region of the limb bud. *Tc Dfd* expression retracts from the mandibular limb bud in *Tribolium* (McGinnis et al., 1998; Brown et al., 1999). In *Glomeris marginata,* there is no expression of *Dfd* in the distal part of the mandible which relates to the developing mandibular endite (Janssen and Damen, 2006). In *Lithobius* there is no *Dfd* expression in the central region of the limb bud (Hughes and Kaufman, 2002b).

However, the dynamics of *dfd* expression are different in other mandibulates. For example, there is no reported retraction of *Dfd* expression from the mandibular appendage in *Oncopeltus fasciatus*[12] or *Thermobia domestica* (Rogers et al., 2002). There is the possibility that the late retraction of *Dfd* expression was not detected because the embryos in the studies in question are too early. In the published *in situ* hybridisation of *Thermobia* there appeared to be a reduction of *Dfd* expression in the central region of the limb (the endite), but this could be an artefact of the plane of focus and the ectodermal expression of *Dfd* (Rogers et al., 2002).

cnc expression Across Mandibulata

Like *Dfd* expression, *cnc* gene expression patterns are highly similar in all studied mandibulates, although the number of taxa sampled is quite small. All mandibulate *cnc* expression patterns consist of two domains, a cap domain in the labrum and a collar domain in the mandibular segment. There is also *cnc* expression around the stomodeum that links the cap and collar domains (Mohler et al., 1991; Mohler, 1993; Mohler et al., 1995; McGinnis et al., 1998; Janssen, 2004; Economou and Telford, 2009).

[12] *Oncopeltus* has a derived mandibular appendage that develops into a stylet so can be ignored for purposes of comparisons to mandibulates.

Other insects in which *cnc* has been studied includes the cricket *Acheta domestica,* the milkweed bug *Oncopeltus fasciatus,* and the firebrat *Thermobia domestica* (Rogers et al., 2002). Outside of insects, only one species has been studied to date, the myriapod *Glomeris marginata* (Janssen et al. 2011). No crustacean homologues of *cnc* have been investigated.

Tribolium

In order to understand the development of the mandible, I chose to study the red flour Beetle *Tribolium castaneum*, a mandibulate which possesses a typical insect mandible and is amenable to functional genetic studies. One obvious question is whether the two genes *cnc* and *Dfd* which are important in *Drosophila* to pattern the mandibular segment play a significant role for development of the mandible in *Tribolium.* Like those of dicondylous insects in general, the *Tribolium* mandible is derived in a number of respects: it is dicondylic and has lost both the telopodite palp and exopodite ramus. But the *Tribolium* mandible possesses the one character that defines the mandible apart from any other appendage type, the gnathal edge made up of the incisor and molar processes on the protopodite of the second post-antennulary limb. The possession of this gnathal edge characterizes the *Tribolium* mandible as resembling the ancestral mandible.

Tc Dfd is necessary to pattern the mandible and maxillary appendages.

Research has demonstrated that the *Tribolium* orthologue of *Dfd, Tc Dfd,* is necessary for patterning the mandibular and maxillary segments in *Tribolium* like in *Drosophila*. In *Tc Dfd* mutants there is a homeotic transformation of the mandible to antenna and loss of the maxillary endite (Brown et al., 1999; Brown et al., 2000). In *Drosophila* however there is no homeotic transformation of the mandible but rather loss of mandibular and maxillary segment derived structures. *Tc Dfd* expression has been shown to retract from the mandibular limb buds. It is therefore interesting to know whether *Tc cnc* plays a similar role in differentiating the mandibular segment from the maxillary segment in part by repression of the maxilla patterning function of *Tc Dfd.*

Another gene, *apontic (apt)* has been shown to pattern the gnathal appendages in *Drosophila* (Gellon et al., 1997). This gene acts in parallel with *Dfd* to pattern ventral

gnathal structures. However, there is no orthologue of the *Drosophila* gene *apontic* in *Tribolium.*

mxp is required to pattern the palp of the maxilla and labial appendages

If *Tc cnc* differentiates the mandible by repressing maxilla development then other maxilla patterning genes may possibly interact with *cnc.* Another Hox gene, *maxillopedia (mxp),* the *Tribolium* ortholog of *pb,* is required to pattern the maxillary palp. Together, *Tc Dfd* and *mxp* pattern the maxillary appendage in an additive fashion (Shippy et al., 2000a). *Tc Dfd* patterns the protopodite, the proximal part of the appendage including the endite, and *mxp* patterns the telopodite, the palp. Mutants of *mxp* possess legs instead of palps in both the maxilla and labial segments. These transformed appendages are attached to a protopodite that is unaffected by the loss of *mxp*.

The orthologue of *cnc* in *Tribolium, Tc cnc*, as mentioned above is expressed in the labral and mandibular segment as well as surrounding the stomodeum like in *Drosophila* (Economou and Telford, 2009). Given that *cnc* is necessary for patterning the mandibular segment in *Drosophila* I was interested in the role that *Tc cnc* might play in patterning the mandibular segment of *Tribolium castaneum* and whether *Tc cnc* represses *Tc Dfd* expression in a manner similar to the mechanism that occurs in *Drosophila.* In order to test the function of *Tc cnc* in *Tribolium*, *Tc cnc* was knocked down using parental RNAi by injecting *dsRNA* into female *Tribolium* pupae (Bucher et al., 2002).

4.2 Results

Tc cnc, Tc Dfd, mxp were amplified by PCR and then subsequently cloned. Antisense labelled RNA probes were synthesized to detect gene expression by *in situ* hybridisation. Double stranded RNA of *Tc cnc* was synthesized to inject into female beetles to knock down *Tc cnc* gene function by RNAi.

Repression of *Tc Dfd* expression from the mandibular endite and appendage in wild type *Tribolium* embryos

Tc Dfd is expressed throughout the mandibular and maxillary segments in the early developing embryo (see fig.4.1A,C-F, fig.4.2A-E, and fig.4.3A,B). As the limb buds start to form, *Tc Dfd* expression progressively retracts from the ventral-proximal part of the mandibular limb buds that relates to the position of the developing endites (see fig.4.3C). Some residual expression remains on the lateral part of the limb bud. During dorsal closure, *Tc Dfd* expression appears to be restricted to the distal portion of the protopodite of the maxillary limb bud (possibly relating to the developing stipes in late stage embryos. *Tc Dfd* expression is absent (or considerably weaker) in the distal part of the maxillary palps throughout embryogenesis (see fig.4.3G,H and fig.4.5E). *Tc Dfd* expression retracts from the developing endites on the limb buds as soon as they form (star in fig.4.3C), which develop on the ventral-proximal region. *Tc Dfd* continues to be repressed (see fig.4.3D-F) until only weak expression of *Tc Dfd* remains on the lateral side of the mandible (see fig.4.3G,H, fig.4.4D,F and fig.4.5C-D).

Using *Tc prd* as a marker for endite development shows that the ventral medial region of the mandibular limb bud where *Tc Dfd* expression is lost encompasses the mandibular endite and the region around it. *Tc Dfd* expression is retained in the lateral part of the mandibular limb bud, but fades throughout embryogenesis (fig.4.5).

Expression patterns of *Tc cnc* have been previously described (Economou and Telford, 2009). *Tc cnc* is expressed in two distinct domains, an anterior cap that includes the developing labrum and stomadeum and a posterior collar domain in the mandibular segment (see fig.4.1B). *Tc cnc* expression remains constant in these two domains from their first appearance during germ band elongation through late embryogenesis (see fig.4.2 and fig.4.3).

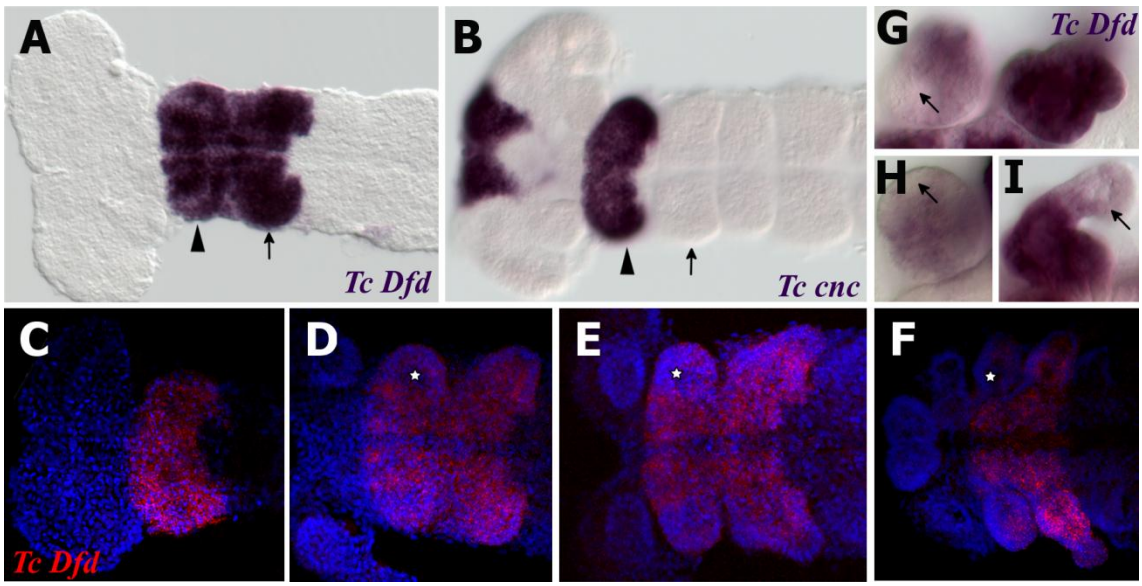

Fig.4.1. *Tc Dfd* and *Tc cnc* expression in the mandibular and maxillary segments of *Tribolium* embryos. All views are ventral with anterior to the left unless otherwise indicated. Gene expression was determined by *in situ* hybridisation. (A) *Tc Dfd* expression in a germ band extending embryo as limb buds are just about to form. Expression is present throughout the mandibular (arrowhead) and maxillary segments (arrow). *Tc Dfd* expression is just beginning to retract from the developing endites (white star). (B) Expression of *Tc cnc* in a germ band extending stage embryo. There is an anterior cap domain of *Tc cnc* in the labrum. The posterior collar domain is indicated with an arrowhead, the maxillary segment is marked with an arrow. (C-F) Fluorescent *in situ* hybridisation of *Tc Dfd* in developing *Tribolium* embryos. White star indicates position of developing endites. (C) Early germ band extending stage embryo prior to limb bud formation. *Tc Dfd* expression is visible in the mandibular and maxillary segments. There is no reduction of *Tc Dfd* expression in the mandibular segment at this stage. (D) Later germ band extending stage than C with retraction of *Tc Dfd* expression evident in the developing endites (white star). (E) Later stage than D with progressive loss of *Tc Dfd* expression. There is no loss of *Tc Dfd* expression in the maxillary protopodite. (F) Germ band retracting stage embryo. *Tc Dfd* expression is almost totally gone from the endite at this stage. Expression that is remaining in the mandibular limb bud is present laterally. (G-I) *Tc Dfd* expression in dissected mandibular and maxillary appendages of germ band retracting embryos. (G) Expression is clearly lost in the inner lobe (arrow) and reduced in the outer lobe. Expression in the maxillary protopodite is ubiquitous. (H) Lateral view. Expression is lost in the inner lobe (arrow), but still present on the lateral part of the mandible. (I) Expression in the maxilla. *Tc Dfd* expression is strong in the maxillary protopodite and faint in the palp (arrow).

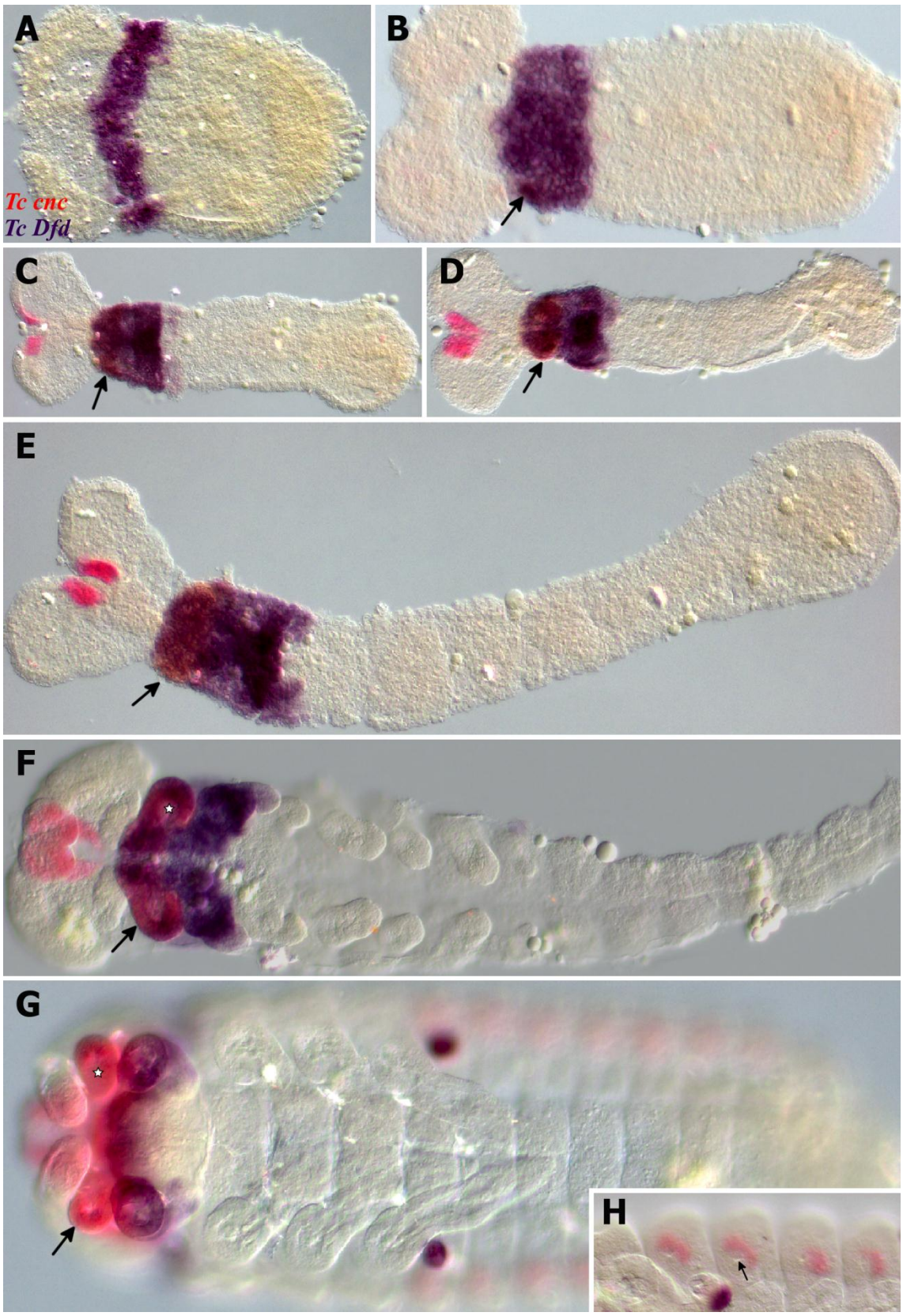

Fig.4.2. Expression of *Tc cnc* and *Tc Dfd* during *Tribolium* embryogenesis. All views are ventral with anterior to the left. Gene expression was determined by *in situ* hybridisation. *Tc Dfd* expression is stained blue, *Tc cnc* expression is stained red. Overlapping domains of expression are brown. Mandibular segment indicated with an arrow. (A-E) Series of germ band extending stages of embryogenesis. (A) Very early germ band extending embryo (the heart stage). (B) early germ band extending embryo. *Tc cnc* is very faintly expressed in the mandibular segment at this stage (arrow). (C-E) *Tc cnc* is expressed in both the developing labrum and the mandibular segment. *Tc Dfd* is expressed

continually throughout the mandibular segment together with *Tc cnc*. The limb buds and the endites have not begun to develop. (F) Fully germ band extended stage. *Tc Dfd* expression retracts from the endites (white star). (G) Fully retracted germ band undergoing dorsal closure. *Tc Dfd* expression is almost completely gone from the mandibular limb bud, with some residual expression residing at the lateral part of the mandible. *Tc Dfd* is strongly expressed in the maxillary segment. The two strongly expressed pair of circular domains of *Tc Dfd* and *Tc cnc* in the first abdominal segment are in the pleuropods. (H) Close up of the abdominal expression domains of *Tc cnc* visible in G. The abdominal domains of *Tc cnc* have a sickle shape which arches around the developing spiracles (arrow).

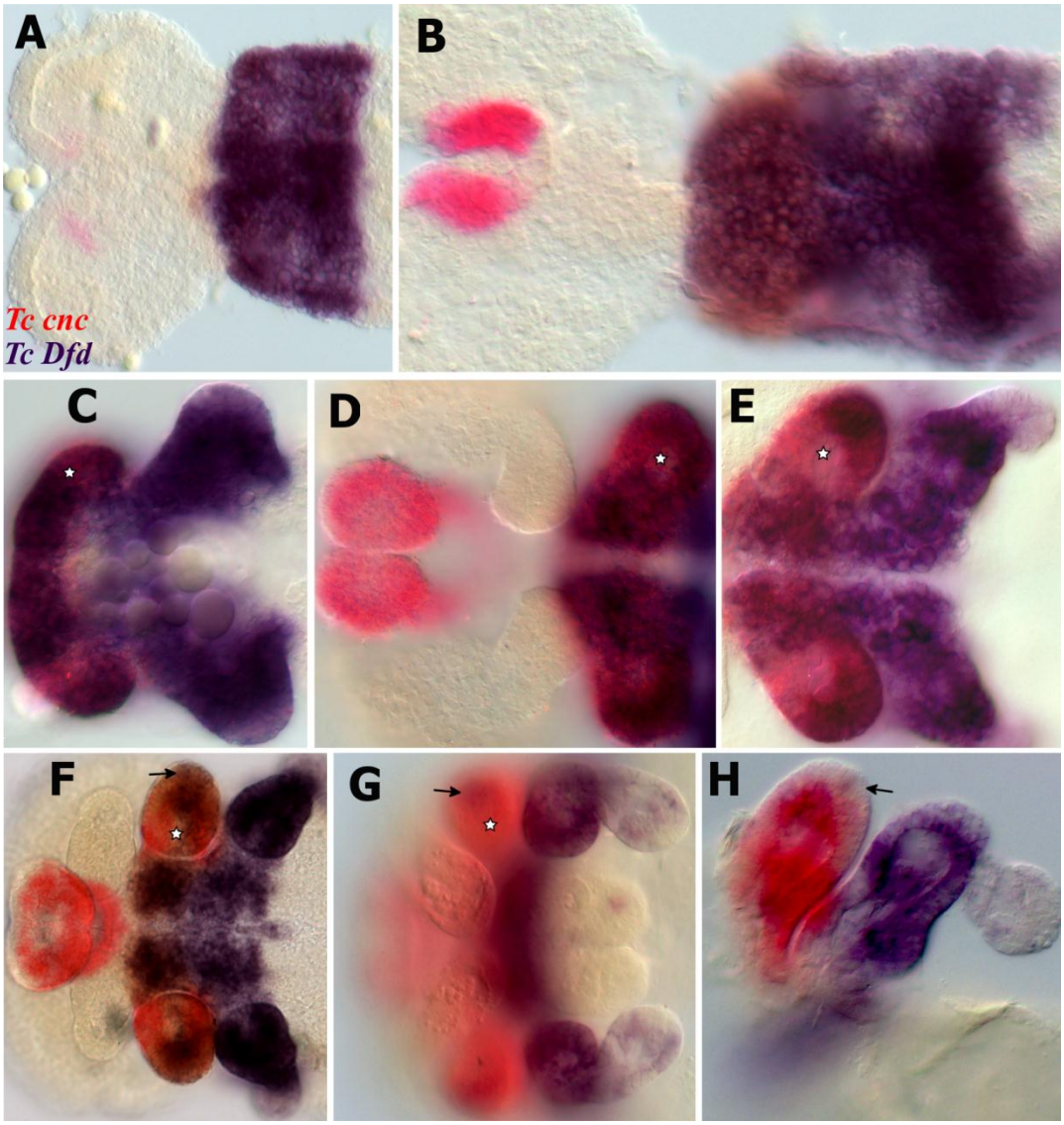

Fig.4.3. Repression of *Tc Dfd* expression in the developing mandibular limb bud. All views are ventral with anterior to the left. Gene expression was determined by *in situ* hybridisation. *Tc Dfd* expression is stained blue, *Tc cnc* expression is stained red. Overlapping domains of expression are brown. As soon as the endites start to form, *Tc Dfd* expression retracts from the mandibular endites (star) whilst *Tc cnc* expression is maintained throughout the mandibular appendage (C-H). (A,B) prior to limb bud formation, *Tc Dfd* expression is continuous throughout the mandibular segment. (C) As soon as the mandibular endite forms, *Tc Dfd* expression retracts from the endite. (G,H) By late embryogenesis, faint *Tc Dfd* expression is only present in the lateral part of the mandibular limb bud (arrow). *Tc Dfd* expression is still strongly maintained in the maxillary segment. (A) early germ band extending embryo. (B) Germ band extending stage embryo prior to limb bud formation. (C) Germ band extending embryo after the limb buds and the endites have started to form. (D) Late germ band extending embryo. (E) Fully extented germ band stage embryo. (F) Germ band retracting embryo. (G) Embryo undergoing dorsal closure with the gnathal appendages moving towards the ventral midline. (H) Lateral view of dissected mandibular and maxillary appendages.

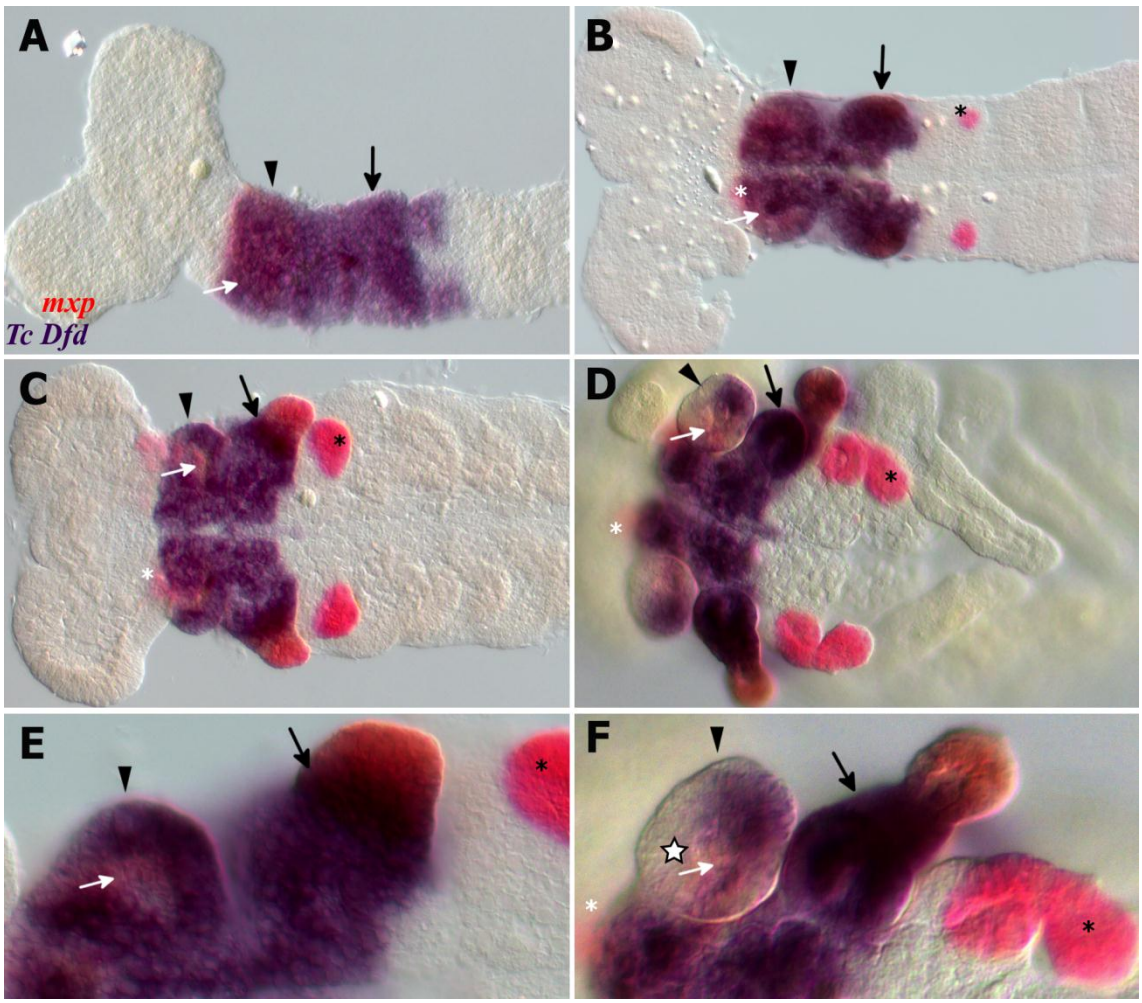

Fig.4.4. Expression of the Hox genes *Tc Dfd* and *mxp* in the gnathal appendage segments during embryogenesis. All views are ventral with anterior to the left. Gene expression was determined by *in situ* hybridisation. *Tc Dfd* expression is blue, *mxp* expression is red, overlapping domains of expression are brown. Mandibular (arrowhead) and maxillary (arrow) segments/appendages and labial palp (asterisk) are indicated. Mesodermal *mxp* expression domain (white arrow) and intercalary domain of *mxp* (white asterisk) are also indicated. (A) Germ band extending embryo prior to the formation of limb buds. *mxp* is expressed in the mesoderm of the mandibular segment. (B) Later germ band extending stage embryo as the limb buds are beginning to form. The maxillary and labial segment developing limb buds are marked by *mxp* expression. *mxp* overlaps with *Tc Dfd* in the maxillary segment (brown) and is solely expressed in the labial segment (red). (C) Later germ band extending stage. The mandibular *mxp* mesodermal expression domain is visible in the mandibular segment (red stain). (D) Germ band retracting embryo. *mxp* expression is present in the palps of the maxilla and labial appendages. (E) Higher magnification of germ band extending embryo (midway between B and C) highlighting the mesodermal expression of *mxp,* the loss of *Tc Dfd* expression in the endite and *mxp* expression in the developing palp of the maxillary segment. (F) Higher magnification image of the gnathal appendages of a germ band retracting embryo as in D. The loss of *Tc Dfd* expression in the endite is shown (star). *mxp* is expressed in the maxilla and labial palps and the distal half of the maxillary and labial protopodites.

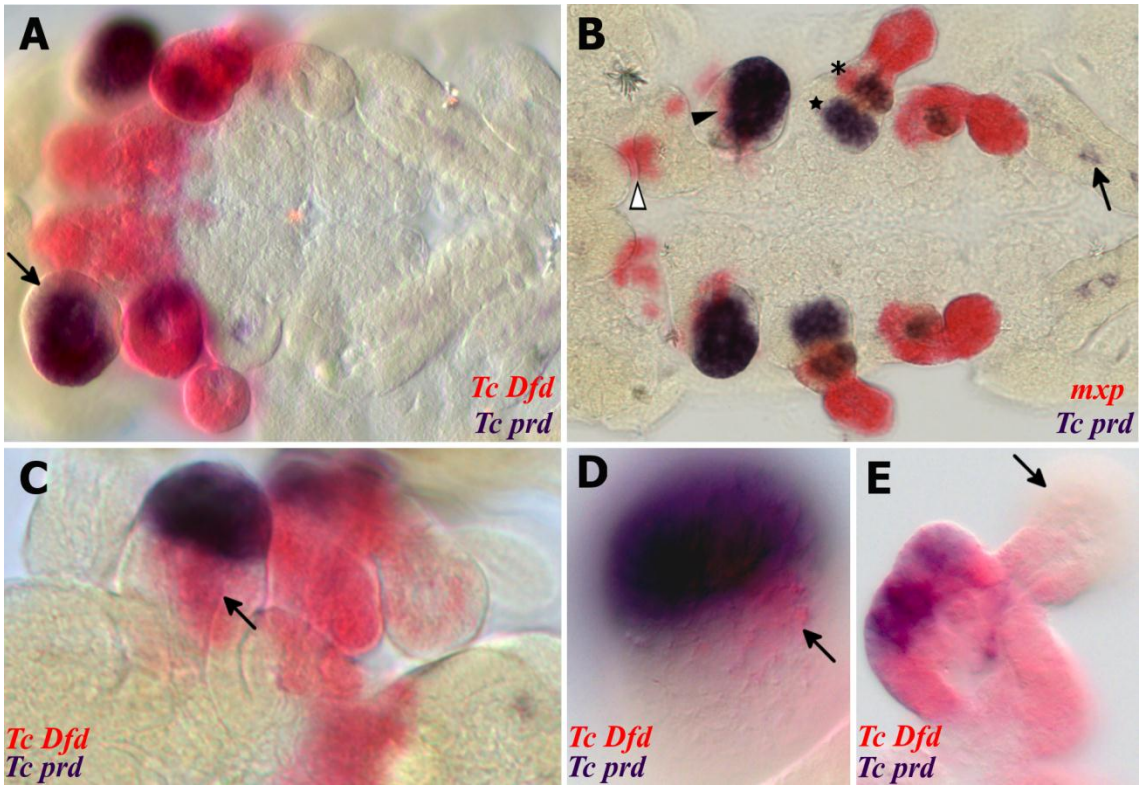

Fig.4.5. Expression of *Tc Dfd* and *mxp* in the mandible and maxilla during embryogenesis relative to the endites marked by *Tc prd* expression. All views are ventral with anterior to the left unless otherwise indicated. Gene expression was determined by *in situ* hybridisation. (A) *Tc Dfd* (red) *and* Tc prd *(blue)* expression in a germ band fully retracted stage embryo. *Tc prd* is expressed in the developing endites of the mandible, maxilla and labial appendages. The proximal part of the inner lobe (arrow) is lacking *Tc prd* and *Tc Dfd* expression. *Tc Dfd* expression is repressed from the developing mandibular endite. (B) Embryo stained with *mxp* (red) and *Tc prd* (blue). *mxp* is expressed in the maxillary and labial palps and the distal protopodite of both appendages. In the maxilla, protopodite expression relates to the position of the developing galea endite lobe (star) which is marked by the distal domain of *Tc prd* expression. The mesodermal mandibular domain is visible underneath *Tc prd* expression (arrowhead). The intercalary domain of *mxp* expression is also visible (white arrowhead). Mesodermal expression of *Tc prd* is present in the telopodites of post-antennal appendages but clearly visible in the developing leg appendages (arrow). (C) *Tc Dfd* expression (red) and *Tc prd* expression (blue) in the mandibular and maxillary appendages of a germ band retracted stage embryo. Lateral view. *Tc Dfd* expression is present on the lateral side of the mandible (arrow). (D,E) *Tc Dfd* expression (red) and *Tc prd* expression (blue) in a dissected mandible and maxilla of a post germ band retracted stage embryo undergoing dorsal closure. Distal is top. (D) Lateral view of a dissected mandible. *Tc Dfd* expression is clearly present on the lateral side of the mandible (arrow). (E) Dissected maxilla, lateral is to the right. *Tc Dfd* expression is throughout the protopodite and at the base of the palp. The distal part of the palp is lacking or has weak *Tc Dfd* expression (arrow).

Tc cnc RNAi phenotype

In order to test the role *Tc cnc* might play in patterning the mandibular segment, *Tc cnc* was knocked down in developing embryos by injecting *Tc cnc* dsRNA into female pupae. Injection of *Tc cnc* dsRNA resulted in homeotic transformation of the mandibular appendages into maxillary identity. Knock down of *Tc cnc* function also resulted in deletion of the labrum. This result shows that posterior collar domain of

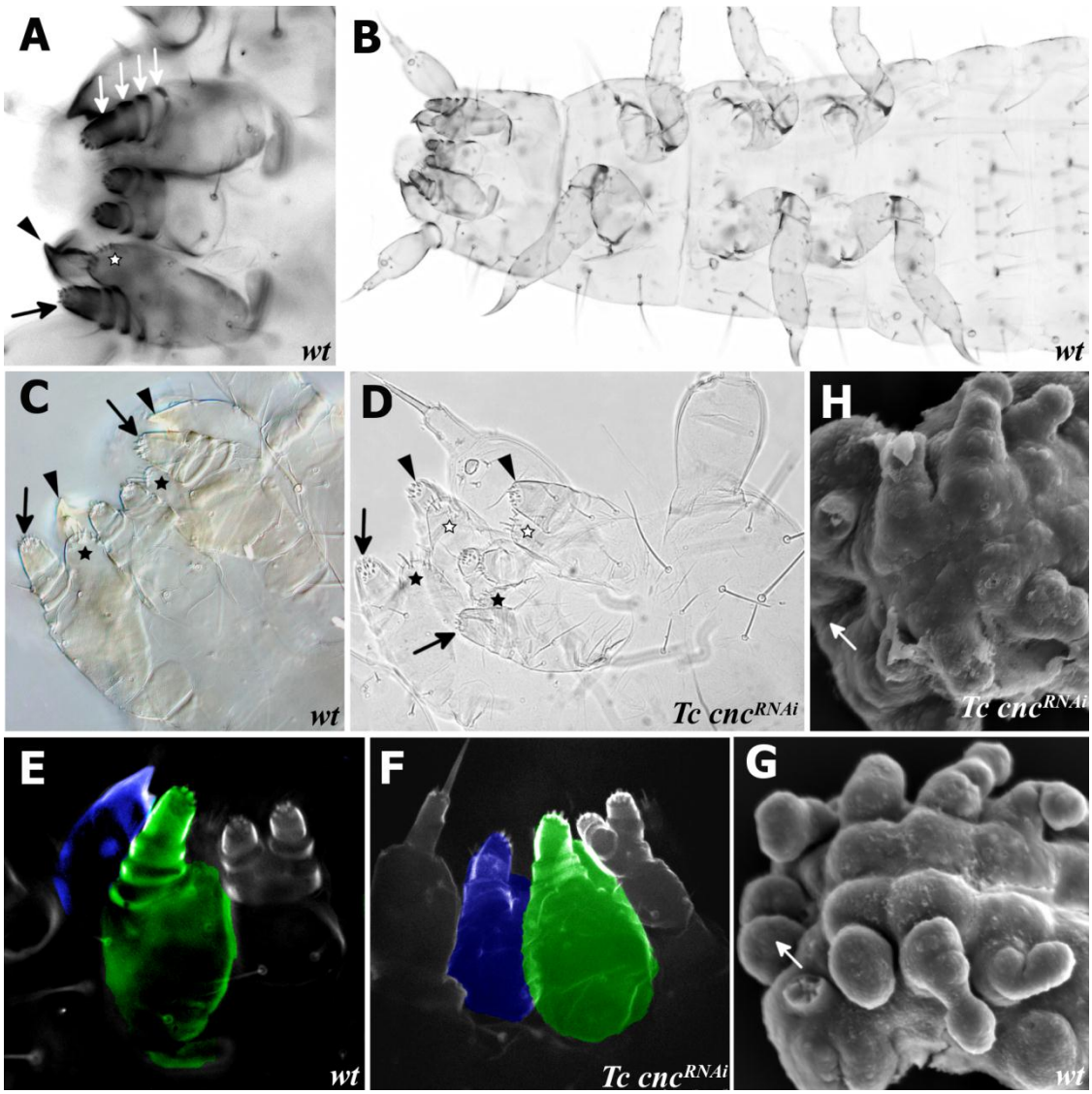

Fig.4.6. *Tc cnc*^{RNAi} results in transformation of the mandibular into maxillary identity and deletion of the labrum. Mandible (arrowhead), maxilla palp (arrow) and ventral branch (star) are indicated. (A-C,F) Cuticle preparations of a wild type *Tribolium* first instar larvae. Cuticle preparations of *Tc cnc*^{RNAi} larvae (D,G). (A) Cuticle preparations of gnathal appendages visualized by fluorescence microscopy. The maxillary appendages have a palp with 4 segments (white arrows) attached to a protopodite with the maxillary endites present. (B) Cuticle preparation of a first instar *Tribolium* larva (C) Cuticle preparation of the larval gnathal appendages of a wild type *Tribolium* larva visualized by DIC microscopy. (D) Cuticle preparation of the gnathal appendages of a *Tc cnc*^{RNAi} larva. Knock down of *Tc cnc* results in transformation of the mandibular appendages into maxillary appendages (arrowheads). The ventral branch is visible on the transformed appendages (white stars). The maxillary appendage is indicated with arrows (palp) and black stars (endite). (E) Cuticle preparation of wild type *Tribolium* larva visualized by confocal microscopy. The mandible appendage is highlighted in blue, the maxillary appendage is highlighted in green. (F) Cuticle preparation of a *Tc cnc*^{RNAi} larva visualized by confocal microscopy. The transformed mandibular appendage is highlighted in blue and clearly resembles the maxillary appendage (highlighted in green) (G) SEM of a wild type embryo at fully extended germ band stage. The labral buds are clearly visible at the anterior of the embryo (arrow). (H) SEM of *Tc cnc*^{RNAi} embryo at germ band extending stage. The labral buds are missing (arrow, compare with G).

Tc cnc differentiates a mandible from a maxillary appendage in the mandibular segment and that the anterior domain of *Tc cnc* performs a gap gene like role in patterning the labrum. This is shown in fig.4.6D,F, where *Tc cnc*^{RNAi} larvae possess an additional pair of maxillae. The mandibular appendages are clearly transformed into maxillary identity, in possession of the maxillary palp with the same number of segments, with the ventral branch (fused lacinia and galea endites in first instar larvae). There is also deletion of the labrum, which demonstrates that the anterior cap domain of *Tc cnc* is necessary to pattern this structure (see fig.4.6H, and fig.4.7B,D-F). There may also be some abdominal defects in some embryos, although it is quite possible that this phenotype was in fact an artefact of the cuticle preparation procedure.

Tc cnc represses *Tc Dll* and modifies *Tc prd* expression in the Mandibular segment.

To investigate the transformed mandibular appendage in *Tc cnc* knockdown embryos, the expression pattern of the homeobox genes *Tc Dll* and *Tc prd* was studied as genetic markers of the developing endites and telopodites.

In wild type embryos, *Tc prd* is expressed in the developing endites of all three pairs of gnathal appendages (see fig.4.5. and fig.4.7A,C). Expression of *Tc prd* is seen in distinct domains of expression in the lacinia and galea enditic lobes of the maxilla, a single domain in the labial enditic lobe and a larger domain of expression in the mandibular appendage.

Tc Dll is expressed in the distal part of all appendages except the mandibular appendage of wildtype *Tribolium* embryos. In the developing maxilla, there are two domains of *Tc Dll* expression, a distal domain in the developing palp and a proximal domain in the lacinia endite. Injecting *Tc cnc* dsRNA results in homeotic transformation of the mandibular appendage into maxillary identity results in complete recapitulation of the maxillary *Tc Dll* and *Tc prd* expression patterns in the transformed mandibular appendage of *Tc cnc* knock down embryos (see fig.4.7B,D-F). The solitary domain of *Tc prd* expression in the mandible is transformed into two domains of *Tc prd* expression that relate to the maxillary enditic lobes. As is expected, transformation of the mandible into maxillary identity results in expression of Tc *Dll* in the palp and proximal enditic lobe (the lacinia enditic lobe).

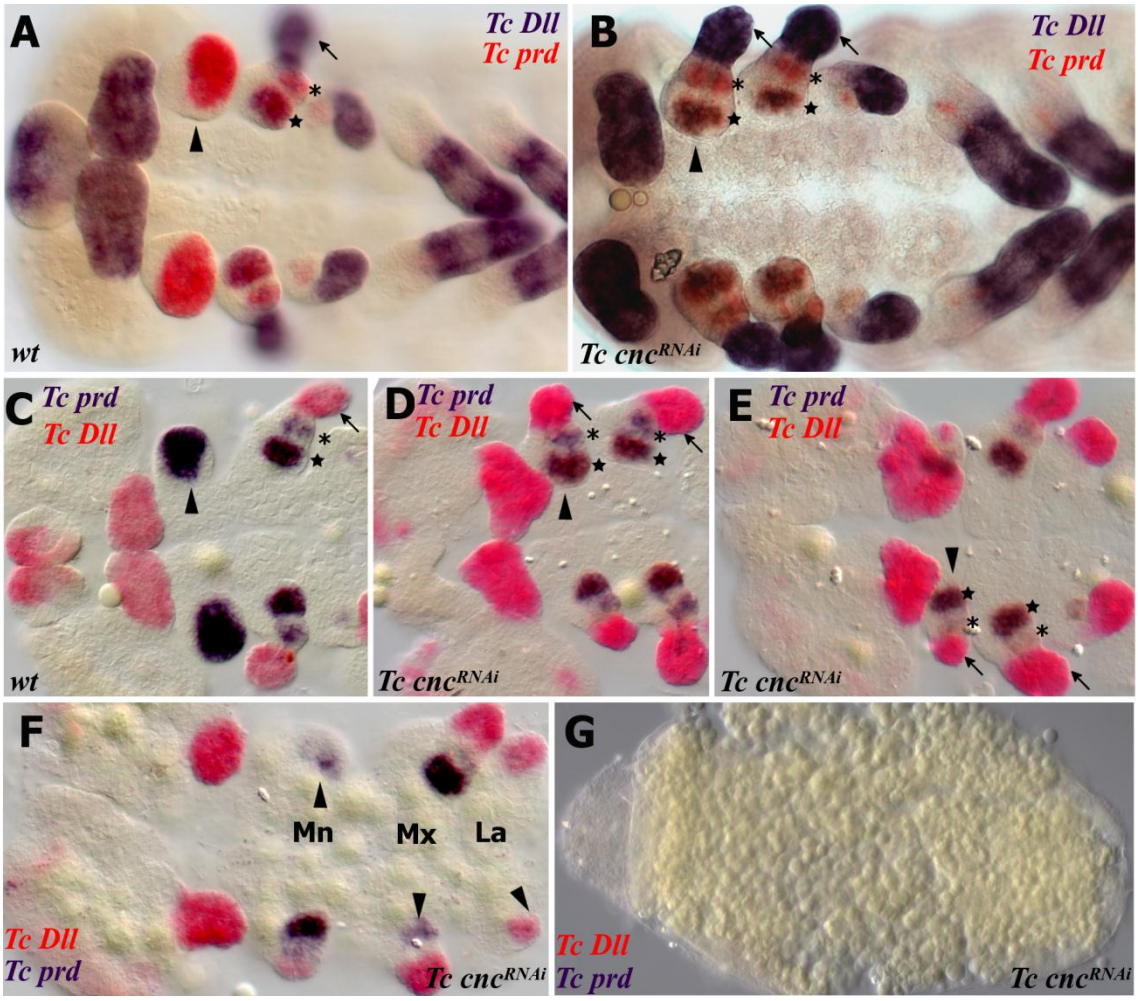

Fig.4.7. Homeotic transformation of the mandibular appendage to maxillary identity and deletion of the labrum in *Tc cnc* knock down embryos as revealed by the expression of *Tc Dll* and *Tc prd.* Gene expression was determined by *in situ* hybridization. All views are ventral with anterior to the left. (A-E) The mandibular appendage (arrowhead), lacinia (star), galea (asterisk) and telopodite (arrow) are indicated. (A,B) Germ band retracted stage embryos that are later than the germ band extended embryos shown in C-E. (A) wild type embryo with the expression of *Tc Dll* (blue) and *Tc prd* (red). *Tc Dll* is expressed in the lacinia endite lobe (star) and the maxillary telopodite (arrow). *Tc prd* is expressed in the endites of the mandible, maxilla and labial appendages. (B) *Tc cnc*^{RNAi} embryo with expression of *Tc Dll* (blue) and *Tc prd* (red). The labral domain of *Tc Dll* is also missing at the anterior of the embryo. These results indicate that *Tc cnc* represses *Tc Dll* and differentiates *Tc prd* expression in the mandible appendage. (C) wild type embryo with the expression of *Tc Dll* (red) and *Tc prd* (blue). (D,E) *Tc cnc*^{RNAi} embryo with the expression of *Tc Dll* (red) and *Tc prd* (blue). (F) *Tc cnc*^{RNAi} embryo stained for *Tc prd* and *Tc Dll.* The telopodite and endite of some appendages are smaller (arrowheads) than their corresponding partner. There is asymmetry between the different transformed mandibular appendages. The transformed mandibles resemble maxillae at an earlier stage of development and so may have delayed development relative to the maxillary appendages. (G) Extreme phenotype observed in a large proportion of embryos. There is a mass of undifferentiated germ tissue concentrated at the polar ends.

The transformed mandibular appendage develops into a maxilla more slowly than the adjacent true maxillary appendages. This is evident by comparing the relative development of the transformed mandibular appendage to the maxillary appendage (see fig.4.7D-F). The mandibular appendage resembles the maxillary appendage at an earlier stage of embryogenesis. This is evident at several stages of embryogenesis.

There appears to be significant asymmetry of different appendages in *Tc cnc* RNAi embryos (see fig.4.7D-F). This asymmetry occurs in a random fashion. This does not appear to be an artefact of the RNAi procedure or the *in situ* hybridisation process as other parental RNAi experiments in *Tribolium* have not yielded a similar result. Instead it seems likely that this is another effect of knocking down *Tc cnc* in *Tribolium*. The reasons for this are unclear, but may be related to the housekeeping role that *cnc* has in protecting the embryo from oxidative stress (Grimberg et al., 2011).

There was an additional strong phenotype observed, where a mass of undifferentiated germ rudiment is present at the anterior of the embryo (see fig.4.7G). It appears that embryogenesis has been seriously affected in these embryos and halted at this stage. The reason for this is unknown. There is no expression of the genes *Tc Dfd, Tc Dll, Tc prd* and *mxp* in the strongly affected embryos. Interestingly, parental injection of *Tc cnc* dsRNA resulted in the mortality of a significant number of the injected females (see materials and methods).

Tc cnc represses the Hox genes *Tc Dfd* and *mxp* in the mandibular limb bud.

The two Hox genes *Tc Dfd* and *mxp* pattern the maxilla in an additive manner (Brown et al., 2000; Shippy et al., 2000b). Consistent with these phenotypes, *Tc Dfd* is expressed in the proximal part of the maxilla, the protopodite, whereas *mxp* patterns the palp (see fig.4.4 and fig.4.5). As there is a homeotic transformation of the mandible into a maxilla, it is to be expected that the Hox genes responsible for patterning the maxillary appendage will be ectopically expressed in the homeotically transformed appendage.

In wild type embryos, *Tc Dfd* expression retracts from the mandibular limb bud (see fig.4.8G,H), which is a likely indication that *Tc cnc* is repressing *Tc Dfd* expression in the mandibular limb bud, in a similar manner to what occurs in *Drosophila*. In *Tc* cnc^{RNAi} embryos, expression of the Hox gene *Tc Dfd* is not repressed from the protopodite of the mandibular appendage which has been transformed into maxillary

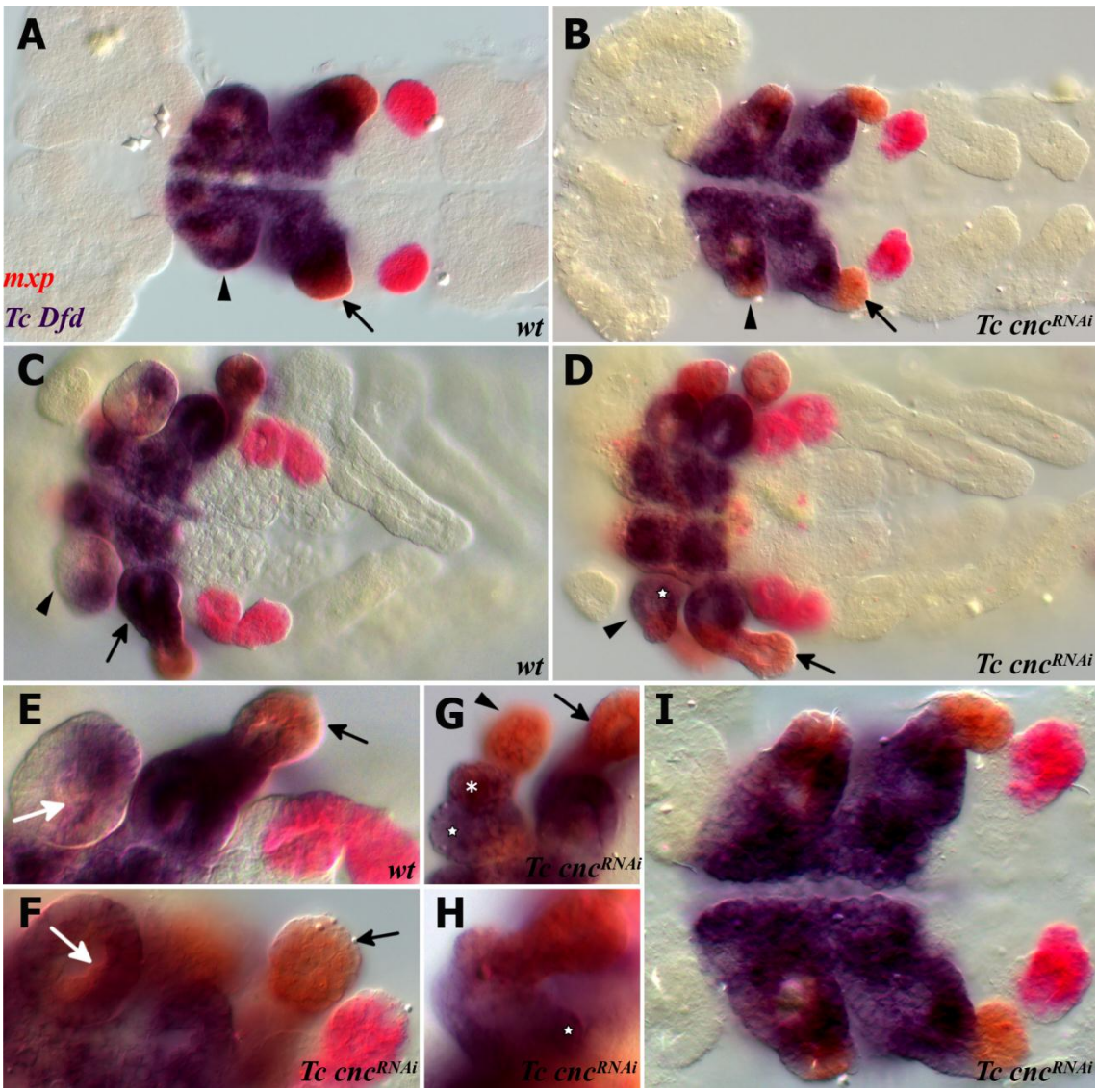

Fig.4.8. *Tc cnc* represses the Hox genes *Tc Dfd* and *mxp* in the mandibular appendage. Knock down of *Tc cnc* by RNAi results in transformation of the mandibular appendage to maxillary identity and the expression of Hox genes in a similar manner to that seen in the maxilla. All views are ventral with anterior to the left. Expression of *Tc Dfd* (blue) and *mxp* (red) is visualized by *in situ* hybridisation. Mandibular segment is indicated with an arrowhead, maxillary segment with an arrow (A,C,E) Wild type *Tribolium* embryos. (B,D,F-I) *Tc cnc*^{RNAi} embryos. (A) wildtype germ band extending stage embryo. (B) *Tc cnc*^{RNAi} embryo at a similar stage to that of A. *mxp* expression is present in the transformed mandibular appendage (arrowhead) in a telopodite domain consistent with the transformation of the mandible to maxillary identity. (C) Wildtype germ band retracting stage embryo. *Tc Dfd* expression has retracted from the majority of the mandibular appendage. (D) *Tc cnc*^{RNAi} embryo at a similar stage to C. *Tc Dfd* expression does not retract from the transformed mandibular endite (star). *mxp* is expressed in the transformed mandibular appendage palp in a manner that is identical to that of the maxilla (arrowhead). (E) Higher magnification of the gnathal appendages of a germ band retracting stage at a similar stage to C, with mesodermal expression of *mxp* (white arrow). (F, G, H) Higher magnification of the gnathal appendages of germ band retracting stage *Tc cnc*^{RNAi} embryos. (F) The mesodermal expression domain of *mxp* (white arrow) is expressed in the transformed mandibular appendage. (G) *Tc Dfd* expression is throughout the transformed mandibular appendage, in the lacinia endite lobe (star), galea endite lobe (asterisk). *mxp* expression is present in the palp as well as the galea endite lobe in a manner which is identical to the maxilla. (H) *Tc Dfd* is expressed throughout the maxilla, the rounded kink at the base of the maxilla is indicated (star). (I) Higher magnification of the earlier germ band extending stage *Tc cnc*^{RNAi} embryo shown in B.

identity. Removal of *Tc cnc* function by RNAi abrogates the repressive effect of *Tc cnc* on *Tc Dfd* transcription. *Tc Dfd* expression is retained in the protopodite (see fig. 4.7A,C,E).

In addition to the repression of *Tc Dfd*, *Tc cnc* also represses (directly or indirectly) *mxp* in the mandibular limb bud (see fig.4.8B,D,F-I). In the transformed mandibular appendage, *mxp* is expressed in the ectopic palp. Interestingly, *mxp* is expressed in the mesoderm of the mandibular segment of wildtype embryos (see fig.4.8F,I) and this mesodermal expression of *mxp* is still seen in *Tc Dfd* knock down embryos (fig. 4.8F,I). This suggests that there is a *cnc* independent regulation of *mxp* in the mandibular limb bud, or that *Tc cnc* is solely expressed in the ectoderm.

Tc Dfd activates the posterior 'collar' domain of *Tc cnc* in the mandibular segment.

In order to investigate whether *Tc Dfd* has any role in the regulation of *Tc cnc, Tc Dfd* was knocked down by parental RNAi and *Tc cnc* expression was detected via *in situ* hybridisation. In Dfd^{RNAi} embryos the posterior collar domain of *Tc cnc* expression is completely missing from germ band extending embryos through to stages where embryos are undergoing dorsal closure (fig.4.9). The anterior cap domain of expression is unaffected. This result shows that *Tc Dfd* is necessary for the activation of the posterior domain of *Tc cnc* in the mandibular segment. The mandibular domain of *Tc cnc* is therefore dependent on the Hox gene *Tc Dfd* for activation.

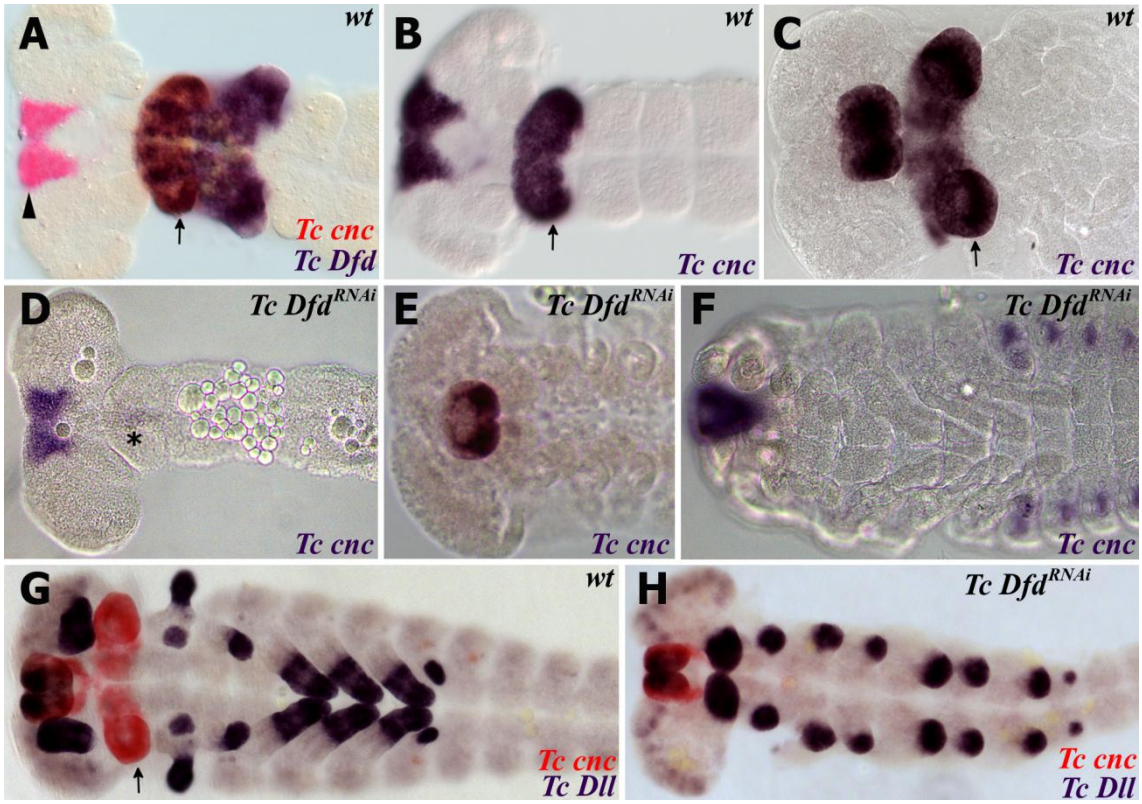

Fig.4.9. *Tc Dfd* activates the posterior collar domain of *Tc cnc* in the mandibular segment. Gene expression was determined by *in situ* hybridisation. (A-C) *Tc cnc* expression in wild type embryos. Throughout embryogenesis, *Tc cnc* expression consists of an anterior cap domain in the labrum (arrowhead) and a collar domain (arrow) in the mandibular segment. (A) *Tc cnc* (red) and *Tc Dfd* (blue) expression in a germ band extending embryo. (B) *Tc cnc* expression (blue) in a germ band extending embryo at a similar but slightly earlier stage to A. (C) *Tc cnc* expression (blue) in later stage embryo prior to dorsal closure. (D-F) *Tc cnc* expression in *Tc Dfd*^{RNAi} embryos. In all stages, from germ band extending (D), germ band retracted (E) and during dorsal closure (F), the posterior domain of *Tc cnc* is missing in the mandibular segment, whilst the anterior domain of *Tc cnc* is expressed as normal. This shows that *Tc cnc* is activated by *Tc Dfd* in the mandibular segment. There is a faint stripe of *Tc cnc* in the mandibular segment of D, this may be due to partial knock down effects. (G) Expression of *Tc cnc* (red) and *Tc Dll* (blue) in wild type germ band retracting embryo. (H) Expression of *Tc cnc* (red) and *Tc Dll* (blue) in a *Tc Dfd*^{RNAi} germ band extending embryo. The posterior domain of *Tc cnc* is missing.

4.3 Discussion

General overview of results

Knock down of *Tc cnc* transcripts by parental RNAi results in a full homeotic transformation of the mandible into maxillary identity and deletion of the labrum in *Tribolium* embryos and first instar larvae. *Tc cnc* represses the Hox genes *Tc Dfd* and *mxp* in the developing mandibular limb bud. This result demonstrates that *Tc cnc* differentiates the mandible from a maxillary appendage in *Tribolium* in part by repressing Hox genes in a manner similar to that observed in *Drosophila.*

Tc cnc^{RNAi} knock down phenotype

Homeotic transformation of the mandible to maxillary identity is seen in the cuticle preparations of knock down larvae and also by the expression of the genes *Tc Dll* and *Tc prd* that are markers for the developing telopodite and endite of the maxilla.

Expression of the gene *Tc Dll* can be seen in the palp and the endite of the transformed mandibular appendage in the developing embryo. *Tc prd* is expressed in both of the developing endites of the transformed mandibular appendage. Both *Tc Dll* and *Tc prd* are expressed in a maxilla-like manner in the transformed mandible. *Tc cnc* therefore represses the *Tc Dll* proximal domain of the endite and distal domain in the palp. *Tc cnc* also modifies *prd* expression domain from that of the two domains of maxillary endites to the large single domain of the mandible endite. Whether these effects are achieved directly or indirectly by *Tc cnc* is not known.

Tc cnc represses the Hox genes *mxp* and *Tc Dfd* in the mandibular segment. The Hox genes *mxp* and *Tc Dfd* are required to pattern the maxillary appendage. The Hox genes *Tc Dfd* and *mxp* are expressed in the transformed mandibular appendage in a manner nearly identical to the maxillary appendage.

There is a mesodermal expression domain of *mxp* in the mandibular mesoderm, which continues to be expressed in the transformed mandible in a *Tc cnc* knockdown embryo. This indicates that the mesodermal expression domain of *mxp* is *Tc cnc*-independent and does not appear to pattern the mandibular appendage.

The ectoderm of the mandibular appendage is fully transformed into an ectopic maxillary appendage. The transformed mandibular appendage mesoderm expresses *mxp* similarly to that observed in wild type mandibular appendage indicating that

there is a *cnc* independent *mxp* domain in the mandibular mesoderm that is unaffected in *Tc cnc* knock down embryos.

These results show that *Tc cnc* is necessary for the development of the mandible, to differentiate it from a maxilla via the repression of two Hox genes and for the activation of genes that are involved in patterning of the mandible. The manner in which *Tc cnc* represses the Hox genes *Tc Dfd* and *mxp* could be direct or indirect.

Significantly, the labrum is also deleted in *Tc* cnc^{RNAi} embryos in a manner analogous to gap genes. In addition there are also possible abdominal effects, in some cuticle preparations of first instar larvae it was apparent that the abdomen was misshapen. Injection of *Tc cnc* dsRNA significantly increases mortality of adult beetles. In *Drosophila cnc* Protein isoform C is known to be involved in responses to oxidative stress (Grimberg et al., 2011).

Comparison with *Drosophila* – similarities

There are numerous similarities between the roles of *cnc* in *Tribolium* and *Drosophila.* In both species *cnc* is required for development of labral and mandibular segment derived structures. With the loss of *cnc* function there are additional maxillary structures in place of mandibular structures, indicating a homeotic transformation has occurred.

The Hox gene *Dfd* is repressed by *cnc* in developing embryos. In *Tribolium*, knock down of *Tc cnc* ablates repression of *Tc Dfd* in the mandibular segment. This result indicates that *Tc cnc* represses *Tc Dfd* transcription and is able to regulate *Tc Dfd* upstream in *Tribolium*. The situation is similar in *Drosophila,* where *cnc* represses *Dfd* in the anterior of the mandibular lobe, with some *Dfd* expression remaining in the posterior of the segment.

Dll is also repressed by *cnc* in both species, although with some differences. The genetic interaction between *cnc* and *Dll* is likely to be indirect. *Dfd* has been shown to activate the proximal domain of *Dll* in *Drosophila* (Regulski et al., 1987; McGinnis et al., 1998; Brown et al., 2000). Repression of *Dfd* by *cnc* prevents activation of this proximal domain of *Dll* by *Dfd* in both species, so *cnc* indirectly represses the proximal domain of *Dll* by inhibiting its activation by *Dfd*.

In *Tribolium Tc cnc* represses the ectodermal expression of *mxp.* In the maxilla *mxp* is expressed in the palp and in the distal part of the protopodite including the

endite of the developing galea. In the transformed mandibular appendage, *mxp* expression is observed both in the palp up to the distal part of the protopodite and the mesoderm.

cnc represses *pb* in *Drosophila,* as the homologue of *cnc* does in *Tribolium*. *pb* is expressed in the distal domain of the maxillary lobe, which is homologous to the distal domain of the *Tribolium* maxillary lobe which becomes the palp. In *cnc* null mutants, there is ectopic expression in the posterior ectoderm of the mandibular lobe, although not a complete recapitulation of the maxillary expression domain of *pb* in the mandible. However, *pb* does not have any discernable function in the *Drosophila* embryo, it is plausible that the expression of *pb* in the distal domain of the maxillary lobe regulated by *cnc* is a remnant of when *pb* did play a role in patterning structures of the distal (or distal) domain such as the maxillary palp.

In both species, there is a *cnc* domain free of *Dfd* expression in the mandibular segment where *Dfd* expression is repressed. In *Tribolium, Tc Dfd* is repressed in the part of the mandibular limb bud which relates to the position of the developing endites. In *Drosophila* the *Dfd*-free domain includes the anterior of the mandibular gnathal lobe and the hypopharyngeal lobes which are situated anterior to the mandibular lobes. In both species, some *Dfd* expression remains in the developing mandibular lobe. In *Tribolium, Tc Dfd* expression remains in the lateral part of the mandibular lobe. In *Drosophila, Dfd* expression remains in the posterior part of the mandibular lobe.

Whether these domains are homologous between *Tribolium* and *Drosophila* is difficult to say, as there are significant morphological differences between the two, in particular the *Drosophila* mandibular lobe is flatter and pressed against the rest of the embryo (in two dimensions). In contrast to *Tribolium* the mandibular lobe is developing into an appendage and is raised above the embryo (in three dimensions). It is possible that these two domains are homologous, one domain with solely *cnc* expression, the other domain with *cnc* and *Dfd* co-expression. But given that fly larvae morphology is highly derived, it is unreasonable to interpret homology on the basis of similar gene expression alone.

More likely is that there is homology between these two domains, but that *Drosophila* embryo has evolved to include new structures such as the hypopharyngeal lobes and lose other structures such as the developing endite lobes.[13]

Comparison with *Drosophila* – differences

In *Drosophila,* loss of *cnc* function does not result in a full homeotic transformation of the mandibular gnathal lobe to maxillary identity. This is in contrast to *Tribolium* where loss of *Tc cnc* function results in a complete transformation of mandible to maxillary identity.

In *Drosophila,* the mandibular gnathal lobe is transformed into the proximal part of the maxillary gnathal lobe, not the entire maxillary gnathal lobe. Structures which derive from the distal part of the maxillary gnathal lobe (like the maxillary sense organ) are not ectopically produced, only proximal derived maxillary structures are produced (like the mouth hooks and cirri). The proximal part of the *Drosophila* gnathal lobe is supposed to be homologous to the proximal part of the *Tribolium* maxillary appendage (Jurgens et al., 1986).

Consistent with the above, only the ectopic maxillary ventral domain of *Dll* is expressed in the mandibular segment of *cnc* mutant embryos, and not the distal domain of *Dll* (McGinnis et al., 1998).

In *Tribolium,* loss of *Tc cnc* functions results in a complete homeotic transformation of the mandibular appendage to maxillary identity. This is evident in the structure of the transformed mandible which resembles a typical maxillary appendage, complete with a palp and endite.

Tc cnc represses both the proximal and distal maxillary domains of *Tc Dll* in the mandible. The proximal domain of *Tc Dll* is activated by *Tc Dfd* (Brown et al., 2000), the telopodite domain of *Tc Dll* is likely to be regulated by the Hox gene *mxp*. *cnc* is regulating the two domains of *Tc Dll* by repressing the Hox genes *Tc Dfd* and *mxp.*

In *Drosophila*¸ the reason for the lack of a full homeotic transformation may stem from the loss of any role of *pb* in patterning the developing embryo. *pb* is

[13] *prd* can be used as a marker for the homologous region in *Drosophila* which gives rise to endites in *Tribolium*: endites are *Dfd* dependent and from the proximal domain. Hypopharyngeal lobes are not homologous to endites as they are from the anteior of the developing mandibular lobe not the proximal part. Therefore the mandibular endites are probably not homologous to the hypopharyngeal lobes.

expressed in the distal region of the maxillary appendage. *cnc* represses *pb* in *Drosophila* embryos, As *pb* does not have any function in the developing *Drosophila* embryo, there is no effect on the distal domain of *Dll* expression or of ectopic distal maxillary structures present in the transformed mandibular lobe. As a result, *cnc* represses *Dfd* and *pb,* but only the proximal maxillary domain of *Tc Dll* is ectopically expressed in the mandibular segment. Only the *Dfd*-dependent domain of *Dll* expression is affected by *cnc* mutants.

In *Drosophila, t*he mandibular segment collar domain of *cnc* is not activated or regulated by *Dfd* or by any other Hox gene (Mohler, 1993). The situation is different in *Tribolium.* The posterior collar domain of *Tc cnc* in the mandibular segment is activated by *Tc Dfd.* In *Tc Dfd* knock down embryos the posterior collar domain of *Tc cnc* is lost. *Tc cnc* requires *Tc Dfd* to pattern all mandibular structures.

In Drosophila, cnc requires *Dfd* to pattern some mandibular structures but is activated independently of *Dfd.* Structures derived from the posterior mandibular segment require *Dfd*. There are other *Dfd*-independent structures which derive from the hypopharyngeal lobes which do not require *Dfd* (Mohler et al., 1995; McGinnis et al., 1998; Veraksa et al., 2000). The significance of the dependence of *cnc* on *Dfd* for patterning mandibular structures in *Drosophila* and whether there are any comparable mechanisms in *Tribolium* is not known.

Another difference between *Drosophila* and *Tribolium* is the presence of hypopharyngeal lobes which are derived from the anterior of the mandibular segment in *Drosophila.* The hypopharyngeal lobes are derived structures that are specific to dipterans and are not present in *Tribolium. cnc* and a gene called *collier (col)* are both required to pattern the hypopharyngeal lobes (Mohler et al., 1995; Seecoomar et al., 2000; Economou and Telford, 2009). *col* has been argued to have a conserved role in patterning the boundary between the intercalary segment and the mandibular segment that is specific to insects (Schaeper et al., 2010). The anterior mandibular domain of *cnc* is upstream of *col* in *Drosophila* (Crozatier et al., 1996; Crozatier et al., 1999; Seecoomar et al., 2000). In *Tribolium,* it has been recently shown that *Tc cnc* is not activated by *Tc col* (Schaeper et al., 2010). Therefore the hypopharyngeal lobe patterning function of *cnc* and the activation of *cnc* by *col* in the anterior mandibular segment of *Drosophila* is a derived condition that reflects the differences between the anterior mandibular segment of dipterans and jaw bearing mandibulates.

The role of *cnc* in mandibulates

In mandibulate arthropods that have been studied, the expression patterns of *cnc* and *Dfd* are highly conserved. In insects and the myriapod *Glomeris marginata*, *cnc* is expressed in two characteristic domains a labral cap domain and a mandibular collar domain. Evidence is lacking from crustacean species. However, considering the phylogenetic position of the crustaceans relative to the insects and myriapods, it is expected that *cnc* will be expressed in a similar manner to both groups.

Dfd is expressed in the mandibular and maxillary segments of all mandibulate arthropods. The expression of *Dfd* is highly conserved across insects. Dynamic expression of *Dfd* expression in the mandibular limb bud, particularly the developing endite, appears to be conserved across mandibulates. The fact that *cnc* expression and the repression of *Dfd* expression from the mandibular endite are widely conserved strongly suggests that the mandibular segment patterning mechanism of *Tribolium* and *Drosophila* is conserved across mandibulates. It remains to be tested whether *cnc* will differentiate the mandible from the maxilla in all mandibulates, particularly crustaceans and myriapods, by repressing the Hox genes which are involved in patterning the maxillary segment.

cnc may function as an activator to pattern mandibular structures

Research in *Drosophila* has shown that despite the importance of *cnc* for repressing Hox gene transcription and function, *cnc* has been shown, as a transcription factor, to function as an activator. The role that *cnc* plays in patterning the mandibular segment in mandibulate insects is likely to be different from *Drosophila* as *Drosophila* has lost the appendage of the mandibular segment. In the mandibular segment of mandibulates such as *Tribolium* that have a mandibular appendage, *cnc* may be required to pattern and differentiate the mandible from the maxilla by the activation of mandible patterning genes.

Comparison with *Drosophila* - Molecular Mechanism of *cnc* regulation of *Dfd*

Tc cnc differentiates the mandible from the maxillary segment and represses the Hox genes *Tc Dfd* and *mxp* in *Tribolium*. This is very similar to what occurs in *Drosophila.* Despite the detailed genetic studies which have been performed in *Drosophila* on the manner in which *cnc* alters *Dfd* transcription and function, the

precise mechanism by which *cnc* functions to repress *Dfd* function and promote development of mandibular segment derived structures is not known. There are however some conclusions that can be drawn, and some implications for the role of *cnc* in mandibulate arthropods (Mohler, 1993; Mohler et al., 1995; McGinnis et al., 1998; Veraksa et al., 2000).

cnc was originally thought to act in a manner similar to a homeotic gene, with *cnc* and *Dfd* acting in a combinatorial manner to pattern the mandibular segment (Mohler et al., 1995; Rogers et al., 2002). As *cnc* represses the expression and activity of *Dfd* in the mandibular segment, *cnc* was reinterpreted to function as a Hox modulator gene (McGinnis et al., 1998). However, detailed experiments to test the role of *cnc* as a transcription factor have surprisingly shown that it functions as an activator and that the repression of *Dfd* is likely to be indirect (Veraksa et al., 2000).

Out of several isoforms, CncB is necessary to pattern mandibular structures and sufficient to pattern anterior mandibular structures derived from the hypopharyngeal lobes. CncB represses both *Dfd* transcription and *Dfd* activity. Repression of *Dfd* activity was detected by studying the effect of CncB on known *Dfd* response elements (DRE), such as the DREs of the *Dfd* autoregulatory loop. However, numerous experiments strongly point to the role of CncB as an activator.

cnc represses the activity of the *Dfd* autoactivation circuit in the anterior mandibular segment, which is necessary for maintenance of *Dfd* expression. Repression of *Dfd* occurs in the anterior of the mandibular gnathal lobe precisely at the same time when the *Dfd* autoactivation circuit is activated in other tissues (McGinnis et al., 1998).

CncB forms a heterodimer with a small Maf protein and binds a *cnc/mafS* binding site sequence (CBS) which is conserved in numerous species. The precise manner in which *cnc* represses *Dfd* is not known. No CBS sequences have been found near the *Dfd* locus, known DREs or any other gene to date. It is therefore likely that CncB represses *Dfd* indirectly.

Veraksa *et al.* have shown that ectopic activation of *cnc* results in a reduction or loss of maxilla segment derived structures and a gain of ectopic hypopharyngeal lobe derived structures (Veraksa et al., 2000). They claim that there are no ectopic mandibular structures as they interpret the hypopharyngeal lobes as derived from the intercalary segment. However, the hypopharyngeal lobes have recently been shown to

be derived from the mandibular segment (Economou and Telford, 2009). In which case, *cnc* does result in the production of ectopic mandibular segment derived structures.

The role of *cnc* in the mandibular segment of mandibulates may include an important role as an activator that patterns specific mandibular segment structures. One direction of research would be to identify which genes are targets of *cnc* in *Tribolium.* One gene that is an obvious candidate based on its mandibular specific endite expression domain is *Tc prd*. In fact, every gene that is differentially expressed in the mandibular compared to the maxillary segment or any appendage type is a candidate for *Tc cnc* regulation.

Molecular mechanism of *cnc* function in *Tribolium*

Tc cnc represses *Tc Dfd* transcription in the mandibular lobe of *Tribolium* just as *cnc* represses *Dfd* in the mandibular segment of *Drosophila*. Based on experiments performed in *Drosophila, Tc cnc* probably represses *Tc Dfd* function in a manner similar to that of *Drosophila*. *Tc cnc* is likely to have an activating function and therefore to repress *Tc Dfd* indirectly. The *cnc/maf* binding sequence (CBS) is highly conserved between diverse taxa although no *cnc* dependent genes have been discovered (Veraksa et al., 2000). There are no hypopharyngeal lobes in *Tribolium*, but there are mandibular appendages with endites, which may be patterned by *cnc*.

It is likely that *Tc cnc* will repress the autoregulatory loop of *Tc Dfd.* Expression of *Tc Dfd* in *Drosophila* activates the autoregulatory loop of endogenous *Dfd* (Brown et al., 1999). The dynamics of *Tc Dfd* expression are similar to those in *Drosophila.*

Expression of other Hox genes in the mandibular segment

The Hox gene *hox3* is expressed in the mandibular segment of some mandibulates outside of higher insects, and may therefore be an important mandible patterning gene. For example Hox3 is expressed in the mesoderm of the developing mandible of the apterygote *Thermobia* (Hughes et al., 2004), the crustacean *Daphnia pulens* (Papillon and Telford, 2007) and the myriapod *Lithobius* (Hughes and Kaufman, 2002b).

However, at present, no *Hox3* homolog has ever been subject to functional analysis, so the role that *Hox3* might play in embryo development is unknown. It is

conceivable that given the diversity of mandibular structures that it may be involved in the development of mandibles of particular organisms.

pb is commonly expressed in the mesoderm of the mandibular segment as well as in the telopodites of developing maxillary appendages. To date no function of *pb* has been discovered that relates to patterning the mandibular segment. In *Tribolium, mxp,* the *Tribolium* ortholog of *Pb,* is expressed transiently in the mesoderm of the mandibular segment during germ band elongation in *Tribolium* (Shippy et al., 2000b), but later fades. *mxp* is also expressed in the mesoderm of the mandibular protopodite. *mxp* mutants do not have any observable mandibular segment defects (Rusch and Kaufman, 2000). *Scr* is also expressed in the mandibular appendage of some species. *Scr* is expressed in the mandibular appendage of *Thermobia* (Passalacqua et al., 2010).

cnc and the evolution of the mandible from a maxilla-like precursor

In some respects, the manner in which *cnc* functions in *Tribolium* and *Drosophila* to differentiate the mandible from a maxilla reflects the evolutionary history of the mandibular appendage. The fossil record indicates that the mandible evolved from a biramous limb and evolved through a maxilla-like appendage. The post-antennal limbs of numerous stem lineage arthropods were serially homologous and undifferentiated from each other. Examples of plesiomorphic serially homologous biramous limbs are present in the Cambrian arthropods *Ercaia* (Chen et al., 2001) and *Martinssonia* (Muller and Waloszek, 1986).

As stem lineage arthropods diverged during the course of evolution in the Cambrian, post-antennal biramous limbs diverged from the primitive biramous limb structure. The second post-antennal segment, homologous to the mandibular segment, evolved from a biramous limb with a well-defined and sclerotized proximal endite similar to those present on the third and fourth post-antennal segments. At some point the characteristic mandibular gnathal edge evolved on the second post-antennal appendage and had differentiated from a maxilla-like precursor into a characteristic mandibular appendage.

If *cnc* shares a similar mandible patterning function by differentiating the mandible from a maxilla across Mandibulata, it suggests that *cnc* acquired a new role to differentiate the mandibular appendage from the maxillary appendage in the ancestor to all mandibulates. The similarities of the expression patterns of *cnc* and *Dfd*

across mandibulates support the notion of a conserved mandible and maxilla patterning mechanism.

It is clear from these results that *Tc cnc* differentiates the mandible from a maxilla by repressing maxilla patterning function of genes such as *Tc Dfd* and *mxp.* The primitive mandible would have resembled the primitive maxilla and possessed two rami, an exopodite-derived ramus and a telopodite-derived ramus (or palp). However, the defining characteristic of the primitive mandibular appendage shared by mandibulate arthropods is the mandibular endite with a molar and incisor process as both the mandible and maxilla possessed palps.

Tc Dfd patterns the protopodite of the maxilla in *Tribolium*. *Tc cnc* differentiates the mandibular protopodite from the maxillary protopodite. This differentiating role of *cnc* to pattern the mandibular protopodite may have been acquired in the ancestor to the Mandibulata.

Considering the similarity of the expression domains in other mandibulates, it is reasonable to expect a similar situation to occur in other species. Given that the mandible has likely evolved from a maxilla-like appendage, the role of *cnc* could have evolved to include a new function to differentiate a maxilla-like precursor into a mandibular appendage.

The mandibular patterning function of *Tc cnc* therefore reflects the history of the evolution of the mandible from a maxilla-like precursor. The new role of *cnc* is likely to include two aspects, that of repressing maxilla patterning Hox genes (such as *Dfd* and *mxp* in *Tribolium*) and that of patterning mandibular specific structures.

To determine whether *Tc cnc* patterns mandibular segment structures it would be necessary to ectopically express *Tc cnc* in another segment in *Tribolium.* This would require the development of genetic techniques such as heat shock transcriptional induction of ectopic gene activation, and mitotic clonal analysis.

The role of *cnc* in repressing telopodite development in the mandible of *Tribolium* is not primitive as the primitive mandibular appendage possessed two rami. If the mandibular differentiating function of *cnc* is conserved in hexapods, the telopodite repressing function (repression of *mxp* in *Tribolium*) has been acquired at some point in the lineage leading to the hexapods. It would be interesting to examine the function of *cnc* in mandibulates that possess mandibular palps in order to see if *cnc* differentiates the protopodite of the mandibular appendage. It would also be

interesting to study the mandibular appendage of myriapods and crustaceans which have also lost the mandibular palps to determine whether *cnc* has convergently evolved a similar function of repression of the telopodite patterning genes in the mandibular segment.

cnc in closely related outgroups to Mandibulata

In closely-related outgroups to Mandibulata, such as the chelicerates and onychophorans there is no obvious differentiation between the first leg appendage and the second leg appendage which are homologous to the mandibular and maxillary segments of Mandibulata (Damen et al., 1998; Telford and Thomas, 1998b; Eriksson et al., 2010). If it is shown that the characteristic posterior domain of *cnc* expression is lacking from the first walking leg segment of these groups, it would provide evidence that the differentiation of the mandibular from the maxillary segment by *cnc* is specific to the mandibulates and is a synapomorphy of that group. This is examined in chapter six.

Tc Dfd patterns the protopodite of the maxilla and the mandible (which is a protopodite structure). Therefore, in order to understand how *Tc cnc* differentiates a mandible from a maxilla protopodite, and what genetic interactions *Tc cnc* is likely to have in the mandible, I studied the genetic interactions of *Tc Dfd* in the maxillary segment. The results of these experiments are shown in the next chapter.

Chapter 5:
The protopodite patterning role of *Tc Dfd* in the maxilla.

5.1 Introduction

The previous chapter demonstrated that *Tc cnc* is necessary to differentiate the mandible from maxillary identity and represses the Hox genes *Tc Dfd* and *mxp,* which are required to pattern the maxillary appendages. In this chapter I wanted to explore the role of *Tc Dfd* in patterning the protopodite of the maxilla, to study the precise morphological phenotype of the affected maxillary appendage and the downstream target genes of *Tc Dfd* in the maxillary protopodite.

Tc Dfd, although repressed by *Tc cnc* in the mandibular segment, is required to pattern the mandibular segment in two key respects; repressing antennal development and activating the posterior collar domain of *Tc cnc* which is required to specify the mandible. Interestingly, in *Tc Dfd* mutants the maxillary appendages lose the endites but otherwise maintain maxillary identity showing that *Tc Dfd* specifically patterns the endites of the maxillary appendage (Brown et al., 1999; Brown et al., 2000). This phenotype suggests that *Tc Dfd* may also have an endite patterning role in the mandibular appendage, as the mandible is in possession of an endite.

However, in *Tc Dfd* mutants, the mandible is transformed to antennal identity in the absence of Hox gene expression (Brown et al., 1999; Brown et al., 2000). As the mandible is transformed into antennal identity, it is not possible to study the endite or protopodite patterning role of *Tc Dfd* in the mandible, as *Tc Dfd* target genes will display antennal specific expression, as well as possible repression of genes that are not involved in patterning the antenna. However, it is reasonable to hypothesize that *Tc Dfd* performs a similar function in the mandibular segment to the maxillary segment and that *Tc cnc* modifies expression of these genes to mandible segment specific domains of expression.

Hox genes pattern the *Tribolium* maxilla in an additive manner

As mentioned above, the *Tribolium* maxilla is patterned by two Hox genes, *Tc Dfd* and *mxp* in an additive fashion. *Tc Dfd* patterns the protopodite and *mxp* patterns the telopodite of the maxilla. Consistent with these phenotypes, *Tc Dfd* is more strongly expressed in the base of the maxilla and weak or non-existent in the palp. *mxp* is expressed in the maxillary palp and the distal part of the protopodite, the galea endite lobe (Brown et al., 2000; Shippy et al., 2000a; Shippy et al., 2000b; DeCamillis et al., 2001).

Dfd expression is conserved in the mandible and maxillary segments of mandibulate arthropods. In particular the expression of *Dfd* is conserved in the protopodites of the maxillary appendages of mandibulates. In addition, expression of *pb* is conserved in the telopodite derived palp of the maxillary segment. The similarity of the expression patterns of these genes suggest that the additive patterning of the maxillary appendage is conserved in diverse mandibulate taxa.

The *Tribolium* maxilla presents an interesting case regarding the role of Hox genes in patterning segments. The additive function of two Hox genes to pattern one appendage type is in contrast to the more typical manner of Hox gene function, posterior dominance. Posterior dominance refers to the dominance of posterior Hox genes over more anterior Hox genes. When a typically more posteriorly expressed Hox gene is ectopically expressed in more segments, the anterior segments take the identity specified by the posterior Hox gene.

Mandible to antenna transformation in *Tc Dfd* mutants

In *Tribolium Tc Dfd* mutants, there is a homeotic transformation of the mandible to antennal identity. The reason for the transformation of mandible to antenna is because Hox genes are needed to repress antennal development. In the absence of Hox genes in the ectoderm of developing appendages, the default appendage type specified is antenna (Brown et al., 2002b). *Tc Dfd* is the only Hox gene which is expressed in the ectoderm of the mandibular segment. In the absence of Hox genes in the mandibular segment in *Tc Dfd* mutants, there is therefore a homeotic transformation of the mandible to antenna as there is no other Hox gene expressed in the ectoderm of the mandibular appendage. The role of Hox genes in repressing antennal development in post-antennal appendages has been argued to represent the ancestral gene function of Hox genes in arthropods (Brown et al., 2000).

The fact that in *Tc Dfd* mutant larvae there is a homeotic transformation of mandible to antennal identity means that analysing the downstream effects of *Tc Dfd* knock down will be complicated by the activation (or de-repression) of the antenna specifying pathway. The antenna patterning pathway involves many of the same genes, such as the PD domain genes and the Notch signalling pathway. Therefore any reduction in expression caused by a loss of *Tc Dfd* will be masked if these genes are activated by the antenna patterning pathway.

For example, genes that are expressed in the antenna, such as *Tc dac*, may be activated by *Tc Dfd* in the mandibular segment. But if those genes are also activated in the antenna specifying pathway then *Tc dac* will continue to be expressed, but as an ectopic antennal domain of *Tc dac*. *Tc Dfd* knock down or mutant phenotypes in the mandible will reveal the genetic interactions which result as a consequence of activating the antenna patterning pathway in the absence of Hox genes.

The *Drosophila* gnathocephalon

The *Drosophila* embryonic gnathal lobes of the mandibular, maxillary and labial segments make up the gnathocephalon. The gnathocephalon does not develop into appendages in the larva, instead it forms the pseudocephalon through a complicated process of cell movements (Jurgens et al., 1986; Diederich et al., 1991). Despite the obvious morphological differences between *Tribolium* and *Drosophila* larvae, the gnathocephalon of *Drosophila* is clearly homologous to the gnathocephalon of less derived insects such as *Tribolium*. The proximal part of the *Drosophila* gnathal maxillary lobe is homologous to the proximal part of the developing *Tribolium* maxilla, specifically the endites (Jurgens et al., 1986). There are also significant similarities in the genetic interactions of mandibular and maxillary segment patterning genes in the proximal region of the maxilla between the two species.

Dfd function in the maxillary gnathal lobe of *Drosophila*

As in *Tribolium, Dfd* is required to pattern proximal maxillary lobe derived structures in *Drosophila* larvae (specifically the cirri, ventral organs and mouth hooks). *Dfd* has been shown to pattern proximal maxillary lobe derived structures by activating *prd, ser* and the proximal domain of *Dll* (Gutjahr et al., 1993; O'Hara et al., 1993; Vanario-Alonso et al., 1995; Li et al., 1999; Wiellette and McGinnis, 1999).

In *Drosophila, Dfd* activates the proximal domain of *Dll* by a maxillary-specific enhancer (called ETD6) and is required for the formation of proximal maxillary lobe derived structures, the cirri (O'Hara et al., 1993). *Dfd* activates the late expression domain of *prd* in the proximal region of the gnathal lobes (Gutjahr et al., 1993; Li et al., 1999). *prd* is necessary for proximal maxillary lobe derived structures (such as the cirri and the ventral organ) (Vanario-Alonso et al., 1995). *ser* is also a target of *Dfd* in the mandibular and maxillary lobes. *ser* is required for normal mouth hook development (Wiellette and McGinnis, 1999).

It is already known that *Dfd* activates the proximal domain of *Tc Dll* in *Tribolium* (Brown et al., 2000). Given that *Dfd* activates *prd* and *ser* in *Drosophila,* I wanted to find out whether *Tc Dfd* activated the orthologues of *prd* and *ser* in *Tribolium.* Regarding *Tc ser,* I wanted to discover whether *Tc Dfd* regulated the formation of maxillary segments in the developing maxillary protopodite by regulating or interacting with the Notch signalling pathway as it does in *Drosophila*.

ser is a component of the Notch signalling pathway required for the formation of arthropod appendage segments. *ser* expression in the gnathocephalon of *Drosophila* is different compared to *Tribolium* as *Drosophila* larvae do not form segmented appendages. Therefore the appendage segment domains of the *notch* signalling pathway are missing from the developing gnathal lobes. In *Drosophila, ser* is expressed strongly in the mandibular segment and the boundaries between each segment of the gnathocephalon (Wiellette and McGinnis, 1999).

There are also differences between late *prd* expression in *Drosophila* and *Tribolium. prd* expression is strongest in the maxillary segment in *Drosophila* and expression fades in the other gnathal segments during embryogenesis, *prd* expression is not obviously associated with developing structures in the *Drosophila* gnathocephalon (Gutjahr et al., 1993). In *Tribolium* late *Tc prd* expression is obviously associated with the developing endite lobes and is strongest in the mandibular endite (Aranda et al., 2008).

Experimental introduction

I was interested in exploring the role that *Tc Dfd* has in patterning the protopodite of the maxilla in more detail with a view to the eventual goal of a more complete understanding of the mandibular and maxillary patterning pathways of

Tribolium. By understanding the precise functions of *Tc cnc* and *Tc Dfd* in patterning the mandible appendage, comparisons across mandibulate taxa will be potentially more informative and conclusions about the homology of mandibular and maxillary patterning mechanisms will be more robust.

Transformation of the mandible into antennal identity with the loss of *Tc Dfd* function means that it is not possible to use RNAi to study the target genes of *Tc Dfd* in patterning the mandibular appendage. However, it is not unreasonable to assume that the target genes of *Tc Dfd* in the maxillary segment will be similar targets of *Tc Dfd* in the mandibular segment, and therefore informative about the role of *Tc Dfd* in patterning the mandibular segment. To demonstrate such claims further work will have to be performed using more complex genetic techniques, such as ectopically activating *Tc cnc,* or using clonal analysis of *Tc Dfd* mutants for example, to understand the mandible and endite patterning function of *Tc Dfd* in more detail. Unfortunately, such techniques are currently unavailable for *Tribolium.*

In order to determine the role of *Tc Dfd* in patterning the maxillary protopodite, *Tc Dfd* was knocked down by RNAi and the effect on other genes was determined by *in situ* hybridisation. *Tc prd* and the proximal domain of *Tc dac* are expressed in both the mandible and maxillary protopodites of *Tribolium* embryos. Both these genes have endite-specific expression domains as described in chapter two. In *Drosophila,* it has been shown that *Dfd* activates *prd* in the maxillary segment. I was therefore interested whether *Tc Dfd* activates *Tc prd* and the proximal domain of *Tc dac* in the maxillary segment.

The mandible and maxilla consist of subcoxal and coxal segments which are marked by *Tc ser* domains of expression as described in chapter three. In *Drosophila,* although there are no larval appendages, *ser* has been shown to be activated by *Dfd.* I wanted to determine whether in *Tribolium Tc Dfd* was required to activate protopodite specific domains of *Tc ser* in the maxillary protopodite.

5.2 Results

Dfd^{RNAi} embryonic and larval phenotype

Knock down of *Tc Dfd* by parental RNAi results in transformation of mandible to antenna identity and loss of the maxillary endites as previously described (Brown et al., 2000). It is evident from analysis of the cuticle preparations of *Dfd*^{RNAi} larvae (fig.5.1C,D) and SEMs of *Dfd*^{RNAi} embryos (fig.5.1I) that the entire endite is missing. The maxillary palp is also affected in in *Dfd*^{RNAi} embryos. In SEMs of *Dfd*^{RNAi} embryos, the affected maxillary palp is larger than the palp of wild type maxilla (fig.5.1H,K,I). This is particularly true of the base of the palp, which in wild type maxillae is quite narrow at the point where it attaches to the protopodite. *Tc Dfd* is expressed most strongly in the protopodite of the maxilla but is also expressed in the base of the maxilla palp, which is consistent with the phenotype observed. In *Dfd*^{RNAi} larvae, the palp and maxilla overall are shorter and smaller than the wild type maxilla (fig.5.1C,D,F).

The mandible is transformed into an appendage of antennal identity, which is smaller than the normal antenna. This is seen in both the SEMs and the cuticle preparations (fig.5.1C,D,I).

Dfd^{RNAi} phenotype of the affected maxillary appendage

There appear to be some segmentation defects in the affected maxilla, and some changes to the identity of some of the sensory bristles (fig.5.2). Sensory bristle morphology and segmentation of the maxilla in *Dfd*^{RNAi} larvae was examined to determine the knock down phenotype in the maxilla. It appears as though there are fewer segments present in the affected maxillae although it is difficult to say this with any degree of certainty as the demarcation between the segments that have been suggested are faint and could alternatively represent distortive artefacts such as a fold or crease from the cuticle preparation procedure. Examination of several affected maxilla reveal the crease to be consistently present, and resembling some true segment boundaries (e.g. the postmentum/prementum segment boundary) and is therefore interpreted to represent a true segment boundary (fig.5.2D-G). The boundaries between segments are hard to decipher in the distal-most part of the affected maxilla. It appears there are two palp segments present, the proximal palp

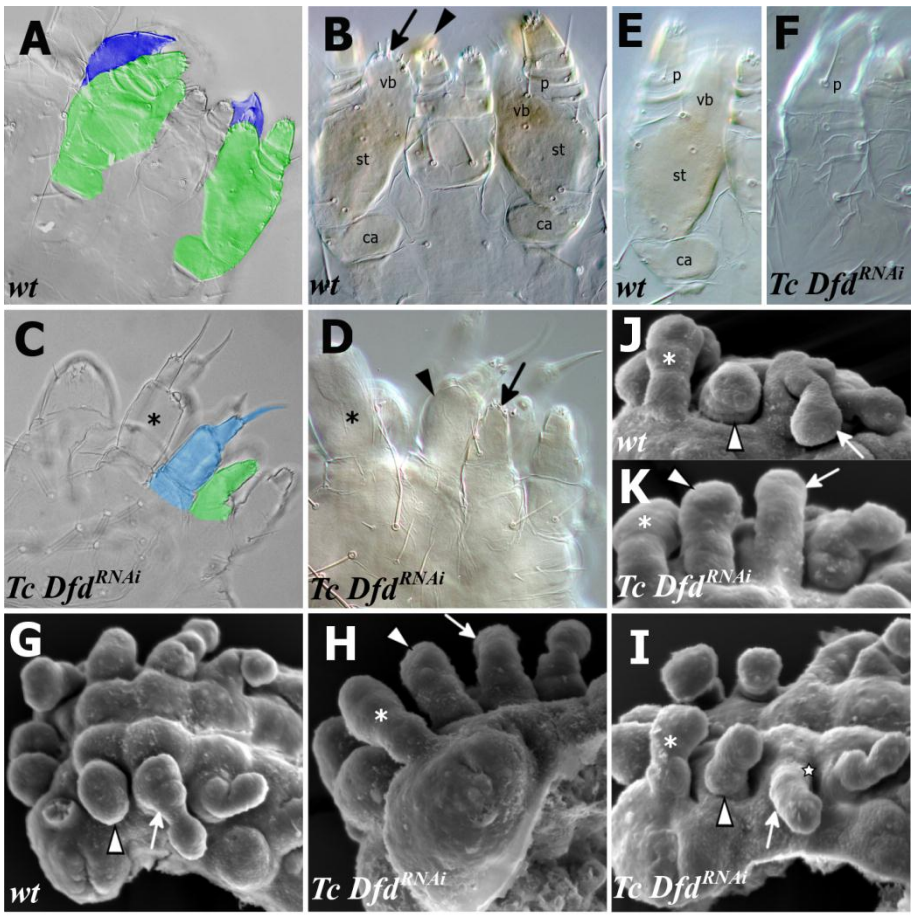

Fig.5.1. *Tc Dfd* patterns the mandible and maxilla in *Tribolium*. SEMs of *Tc Dfd* knock down embryos and cuticle preparations of first instar larvae show the transformation of the mandible (arrowhead) to antennal identity and alteration of maxilla morphology, particularly regarding the protopodite. All views are ventral with anterior to the left unless otherwise indicated. Maxilla is indicated with arrows. The antenna is indicated with asterisks. (A) Wild type cuticle preparation of *Tribolium* larval head showing the mandibular appendages highlighted in blue and the maxillary appendages highlighted in green. (B) Wild type cuticle preparation of *Tribolium* larval head showing the maxillary and labial appendages. The mandible (arrowhead) is out of focus. (C) *Tc Dfd*^{RNAi} larva with the transformed mandibular appendage highlighted in green and the affected maxillary appendage highlighted in blue (compare to A). The transformed mandible clearly resembles the antennal appendage, although smaller in size. The maxilla is shorter and has lost the ventral branch endite. (E) Close up of the maxilla of a wild type larva. (F) Close up of the maxilla of a *Tc Dfd*^{RNAi} larva. (G) SEM of a wild type embryo at the germ band retracting stage. The mandible, (inner and outer lobes), maxilla and labial endites are visible. (H) SEM of a *Tc Dfd*^{RNAi} larva at a similar stage to G, lateral view. The mandible has been transformed to an antenna. (I) SEM of a *Tc Dfd*^{RNAi} larva at a similar stage to G. Ventral-lateral view. The endites of the maxilla have clearly been lost (asterisk). The transformed mandible resembles the antenna, but in a smaller form. (J) SEM of the gnathal appendages of a wild type embryo at a similar stage to G. Lateral view. (K) SEM of the anterior appendages of a *Tc Dfd*^{RNAi} embryo at a similar stage to G. Ventral-lateral view. The mandible has antennal morphology. The affected maxilla is uniform and relatively undifferentiated along the P/D axis compared to the wild type maxilla (compare with J). Abbreviations are as follows: cardo (ca), stipes (st), palp (p) and ventral branch (vb).

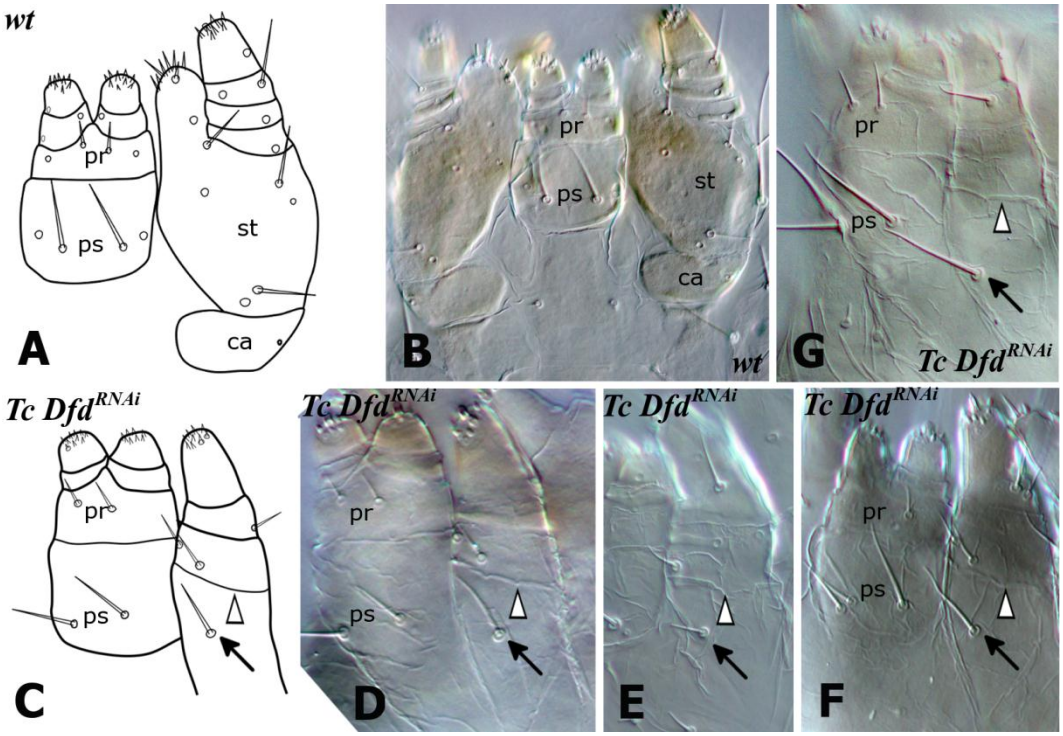

Fig.5.2. Loss of *Tc Dfd* function affects segmentation and bristle morphology in the larva maxilla. All views of appendages are distal to the top, larval head orientation is anterior to the top. (A) Schematic of wild type larval maxilla and labial appendages derived from B. (B) Wild type cuticle preparation of *Tribolium* larval head showing typical segmentation and bristle morphology of the maxilla and labial appendages. (C) Schematic of *Tc Dfd*^{RNAi} larva maxilla and labial appendages. Arrowhead indicates the putative subcoxa/coxa segment boundary, the arrow indicates the labial appendage-like subcoxa bristle present near the base of the affected maxilla. Compare with D. (D-G) Four maxillae from different larval cuticle preparations. The affected maxillae are shorter and missing the ventral branch endites. The putative subcoxa/coxa segmental boundary (white arrowhead) and labial-like proximal bristle (arrow) is visible in each appendage. There is no apparent subcoxa or cardo boundary with the head, it appears as though the affected maxillary appendage is continuous with head cuticle. The palp of the affected maxilla appears to consist of two segments. However, the cuticle preparations are not of sufficient quality to confirm this interpretation. It is clear that the distal most segment of the palp resembles the more elongate maxillary palp and not the shorter, stubby labial palp.

segment having a bristle present on it which is similar to that present on the typical maxilla palp. In light of the above, the affected maxilla is interpreted to have four segments, a two-segmented palp attached to a two-segmented protopodite. This is in contrast to a wild type maxilla that has two protopodite segments and a palp consisting of four segments.

The pattern of bristles on the affected maxilla is also different from the wild type maxilla. There are similarities of the affected maxilla to half the labial appendage (which consists of two fused maxillae). Superficially, the affected maxilla looks as

though it has transformed into a labial appendage without fusing with the protopodite of the other affected maxilla. However, the distal-most segment of the palp of the affected maxilla resembles the palp of the normal maxilla and not the labial appendage. There is other genetic evidence in favour of maxilla identity of the affected palp which is discussed below. The pair of large bristles present on the postmentum of the labial appendage are in a similar position to the bristles present in the affected maxilla and is interpreted to represent the distal-most segment of the protopodite, the coxa.

In the wild type maxilla, there are three main bristles present on the stipes. In the transformed maxilla, there are three bristles in a similar position, however the proximal bristle is somewhat enlarged and therefore appears to have been altered by *Tc Dfd* knock down (arrow in fig.5.2C). There is an apparent segment boundary between the proximal bristle and the other two bristles, which is different from the normal maxilla. The cardo/stipes segment boundary which has a characteristic angular morphology in the wild type maxilla is not recognizable in the affected appendage, which has a more uniform telescopic appearance.

With consideration of gene expression data shown below, the protopodal segment boundary in the affected maxilla (triangle in fig.5.2C-G) is suggested to represent the altered cardo/stipes boundary of the normal maxilla, which is interpreted in this work as the subcoxa/coxa boundary present in post-antennal appendages. The bristle that is present in the subcoxa segment is a bristle that is repressed by *Tc Dfd* and not normally found in the subcoxa (the cardo) of the wild type maxilla.

Expression of the Hox gene *mxp* in *Dfd*^{RNAi} embryos

In order to confirm that the maxillary Hox gene is still expressed in the maxillary segment and that the remaining palp was of maxillary identity, *mxp* was detected in *Dfd*^{RNAi} embryos. *mxp* is expressed in a domain that includes the telopodite and extends proximally to the galea enditic lobe. *mxp* is not expressed in the proximal part of the protopodite (fig.5.3A). Expression of *mxp* did not appear to be significantly affected in *Dfd* knock down embryos; *mxp* is still expressed in the palp of the affected maxillary appendage as in wild type embryos. In *Tc Dfd*^{RNAi} embryos, the posterior collar domain of *Tc cnc* is lost (see chapter four). Therefore, *Tc cnc* was detected

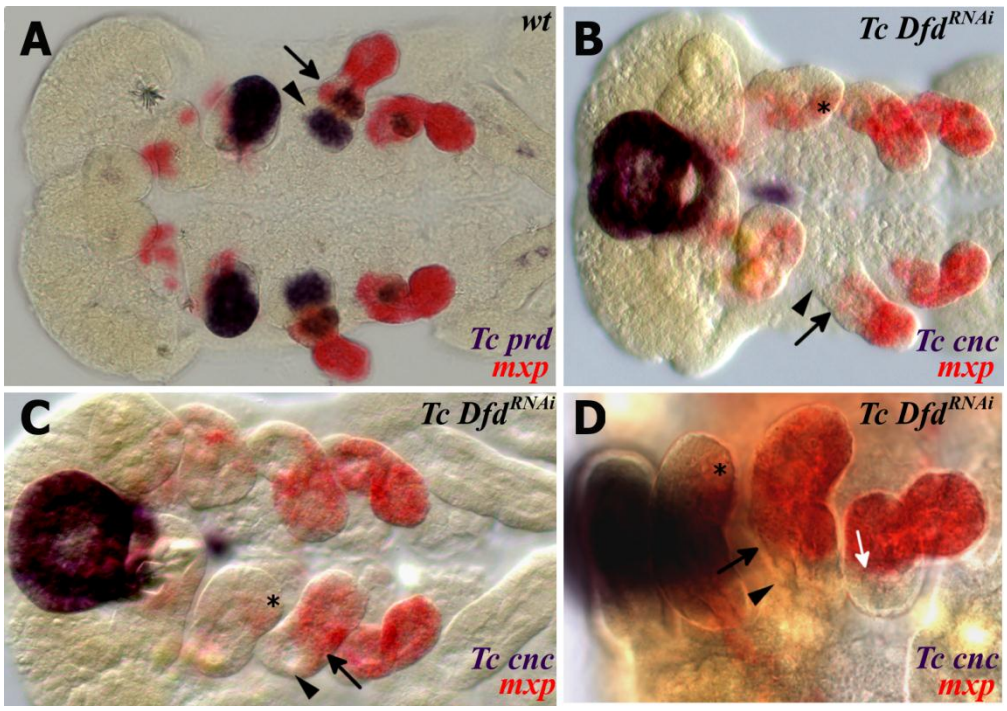

Fig.5.3. Maxillary palp identity is maintained in the absence of *Tc Dfd* function. *Tc Dfd* does not activate *mxp* in the maxillary segment. Gene expression was detected by *in situ* hybridisation (A) Wild type germ band retracting stage embryo stained for *Tc prd* (blue) and *mxp* (red). *Tc prd* is expressed in the endites of the gnathal appendages. In the maxilla there are two domains, a lacinea endite domain (arrowhead) and a galea endite domain (arrow). *mxp* expression is present in the palps of the maxillary and labial appendages. Expression is present in the distal part of the protopodite that relates to the galea endite. (B-D) *mxp* expression remains in the palps of the labial and affected maxillary palps. There is a proximal region of the affected maxilla that is lacking *mxp* expression (arrowhead).The proximal limit of *mxp* expression (arrow) and the region free of *mxp* expression in the affected maxilla is in a similar position to the labial appendage (white arrow in D) which is unaffected by *Dfd* knock down. This suggests there is no deletion of a proximal region of the P/D axis. The posterior domain of *Tc cnc* in the mandibular segment is missing in *Tc Dfd*^{RNAi} embryos. Expression of *mxp* is present in the posterior of the transformed mandibular appendage (asterisk). (B) Expression of *mxp* (red) and *Tc cnc* (blue) in *Tc Dfd* knock down embryo at late germ band retracting stage. (C) Expression of *mxp* (red) and *Tc cnc* (blue) in a later stage (germ band retracted stage) *Tc Dfd* knock down embryo than B. In both B and C there is a small spot of *Tc cnc* in the ventral midline, the significance of this is not known and has been observed in some *Tc Dfd*^{RNAi} embryos. (D) Close up of the expression of *mxp* (red) and *Tc cnc* (blue) in the anterior appendages of a *Tc Dfd* knock down embryo at late germ band retracting stage. The anterior cap domain of *Tc cnc* is visible out of focus as a dark blue blur at the anterior of the embryo.

simultaneously in doubly stained *Tc Dfd* knock down embryos to confirm that *Tc Dfd* was knocked down by the lack of *Tc cnc* expression in the mandibular segment (fig.5.3B-D).

Whilst the loss of the maxillary endites is quite obvious, it is difficult to tell from cuticle preparations of knock down first instar larvae or scanning electron micrographs of knock down embryos whether the protopodite has been deleted or is merely reduced in size. It is also difficult to determine whether there is a cardo/stipes segmentation boundary present. By studying the expression patterns of several marker genes that are normally expressed in the telopodite or the protopodite, it is possible to

determine whether there has in fact been any obvious deletion of the protopodite in a *Tc Dfd* knock down embryo.

Examination of the expression of *mxp* in *Tc Dfd* knock down embryos reveals that there is still a proximal region that lacks *mxp* expression which is in a similar position to *mxp* expression in the labial appendage, which shows that there is still a protopodite present (fig.5.3B-D). Expression of *mxp* in the labial appendage marks the position of the endite possessing prementum segment. Expression of *mxp* in the maxillary appendage of Dfd^{RNAi} embryos is in similar position (white arrow in fig.5.3D). *mxp* expression is in the distal half of the protopodite which is consistent with no deletion of the protopodite.

There is faint expression of *mxp* in the ectoderm of the posterior half of the mandibular appendage (see fig.5.3B-D). The significance of this is not known, and is not expected either as the transformed mandible is of antennal identity which is typically repressed by the presence of a Hox gene.

Tc Dfd upregulates the proximal domain of *Tc dac.*

Tc Dac is expressed in two domains in the maxilla, a stronger proximal domain that is located in the protopodite with a particularly strong ring of expression around the protopodite located on the distal margin of the lacinea lobe (arrow in fig.5.4A). The distal domain is weaker and is located in the middle of the palp (arrowhead in fig.5.4A). In *Tc Dfd* knock down embryos, both domains are still present. However, the proximal domain is significantly weaker (fig. 5.4B-D). There is an inversion in the relative strength of expression of these domains, as the distal domain is significantly stronger than the proximal domain which is reminiscent of the expression pattern seen in the legs. The strong expression domain of *Tc dac* in the mandible is lost. Expression of *Tc dac* in the transformed mandible (asterisk in fig.5.4C) resembles the *Tc dac* expression domain of the antenna. The reduction of *Tc dac* expression in the affected maxilla of Dfd^{RNAi} embryos indicates that *Tc Dfd* up-regulates expression of the proximal domain of *Tc dac*.

Tc Dfd patterns the endites of the mandible and maxilla

Further analysis of the protopodite of the affected maxillary appendage of a Dfd^{RNAi} embryo was conducted by studying the expression of *Tc Dll* and *Tc prd*. The expression of *Tc Dll* in *Tc Dfd* mutants has previously been described (Brown et al.,

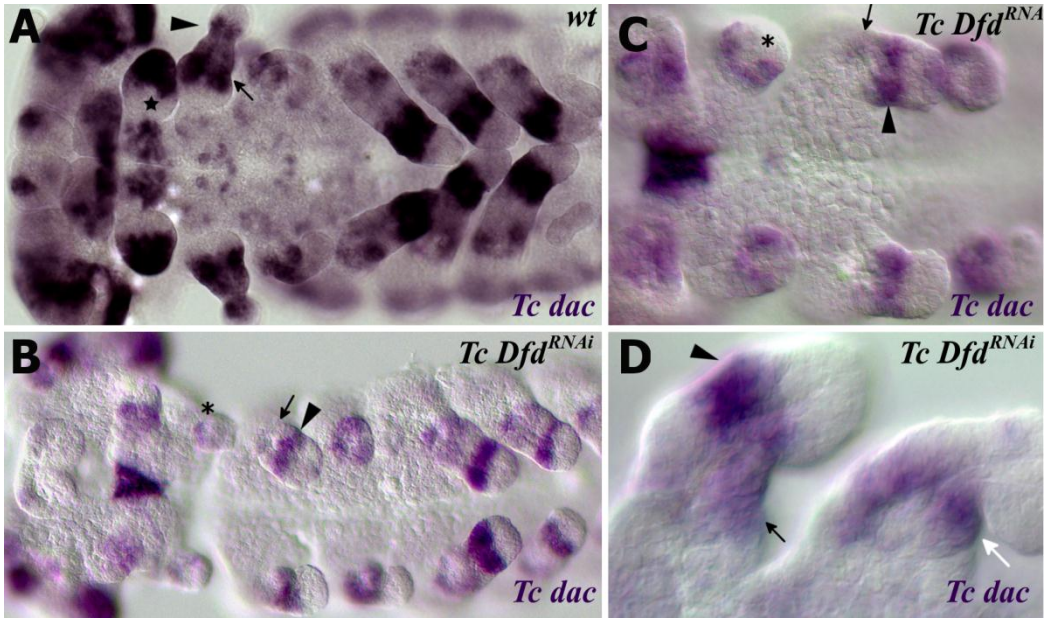

Fig.5.4. *Tc Dfd* upregulates the proximal domain of *Tc dac* in the maxilla. Gene expression was detected by *in situ* hybridisation. (A) Expression of *Tc dac* in a wild type germ band retracting stage embryo. *Tc dac* is expressed in two domains in the maxilla, labial and leg appendages. There is a proximal domain (arrow) and a distal domain (arrowhead). The gnathal appendages have large proximal expression domains. In the legs, the proximal domain is faint and the distal domain in the develping femur and tibia is markedly stronger. (B-C) Expression of *Tc dac* in *Tc Dfd*^{RNAi} germ band extending stage embryos. The mandible is transformed into an antenna (star) and has lost the mandible proximal expression domain. The proximal domain in the maxilla is faint. Conversely, the distal domain is larger. The proximal domain of the labial appendage is unaffected. (C) Close up of the expression of *Tc dac* in the gnathal appendages of a *Tc Dfd*^{RNAi} germ band extending stage embryo. (D) Close up of the expression of *Tc dac* in the affected maxilla and labial appendage of a *Tc Dfd*^{RNAi} germ band extending stage embryo. Here the reduction in expression of the proximal domain of *Tc dac* (arrow) relative to the distal domain (arrowhead) can be seen clearly. The labial proximal domain (white arrow) is considerably larger than the distal domain (white arrowhead).

2000). The proximal domain of *Tc Dll* in the developing lacinea enditic lobe is lost, which indicates that the lacinea endite lobe is lost. In *Tribolium Dfd*^{RNAi} embryos, *Tc Dll* is expressed in the distal part of the affected maxilla appendage. *Tc Dll* expression is completely lacking from the proximal region of the maxilla which corresponds to the protopodite.

To analyse the role of *Tc Dfd* patterning the endites of the maxilla in more detail, an endite marker gene *Tc prd* was studied in *Dfd*^{RNAi} embryos. *Tc prd* is expressed in all endites of the gnathal appendages (fig.5.5A,B). Detection of *Tc prd* transcripts show unambiguously that the mandibular and maxillary endites have been lost in *Tc Dfd* knock down embryos (asterisk in fig.5.5C,D). The endites of the labial appendage are still present in *Dfd*^{RNAi} embryos, with the expression of *Tc prd* marking the labial endite (star in fig.5.5C-D). There is no expression of *Tc prd* in either the

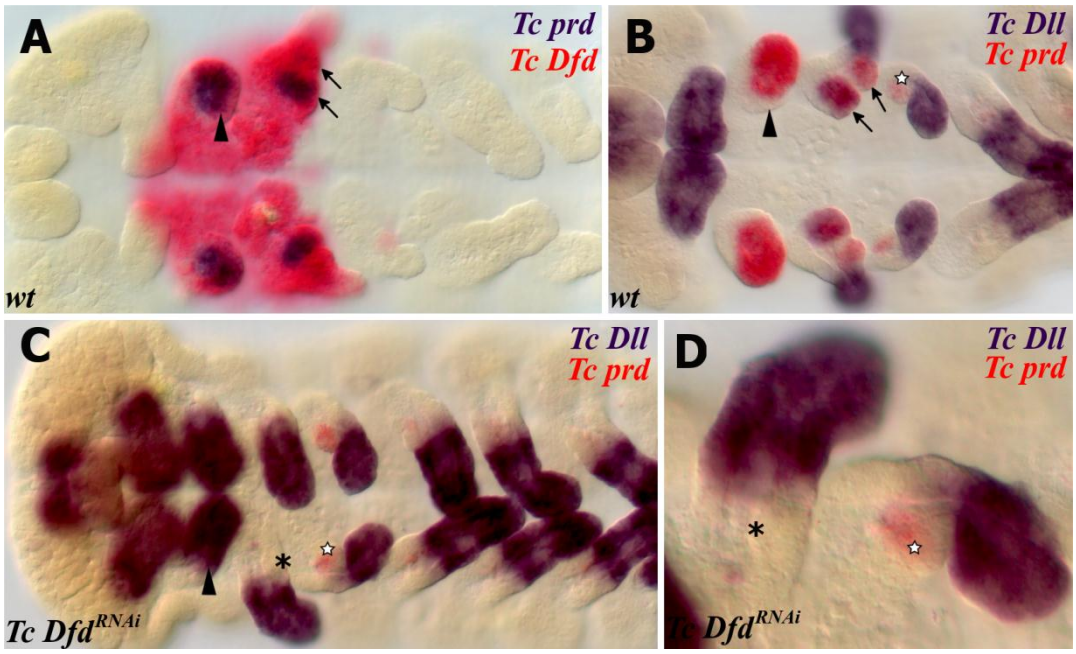

Fig.5.5. *Tc Dfd* patterns the endites of the mandibular and maxillary segments. Gene expression was detected by *in situ* hybridisation. The mandibular segment appendage is marked with an arrowhead. The maxillary endites are marked with arrows in wild type embryos (A, B). (A) Expression of *Tc prd* (blue) and *Tc Dfd* (red) in a wild type germ band extending embryo. *Tc prd* is expressed in the developing endites of the gnathal appendages. *Tc Dfd* is expressed in the mandibular and maxillary segment embryos. (B) Expression of *Tc prd* (red) and *Tc Dll* (blue) in a wild type germ band extending embryo. *Tc Dll* is expressed in the lacinea endite lobe. There is no *Tc Dll* expression in the mandible (arrowhead). (C) Expression of *Tc prd* (red) and *Tc Dll* (blue) in a *Tc Dfd*^{RNAi} germ band extending embryo. The mandible has been transformed into an antenna which expresses *Tc Dll* (arrowhead). There is no maxillary endite (asterisk). The labial appendage has an endite (star) marked with *Tc prd* expression. (D) Enlargement of the altered maxilla in C. There is no *Tc prd* expression in the affected maxilla. There is a significant region of the base of the affected maxilla excluding *Tc Dll* expression which indicates that the protopodite is still present.

transformed mandible or the affected maxilla. This result shows that *Tc Dfd* is necessary to pattern the endites of the mandible and maxilla.

Segmentation is unaffected by loss of *Tc Dfd* function in the protopodite.

A lack of expression in the proximal part of each limb for both *mxp* and *Tc Dll* and the presence of the proximal domain of *Tc dac* shows that there is still a protopodite in *Tc Dfd* knock down embryos. It is not obvious that the proximal segments have either been preserved or lost in the affected maxilla in *Tc Dfd*^{RNAi} embryos or larvae (fig.5.2C-F). In order to investigate the role of *Tc Dfd* in patterning the segments of the protopodite, the expression pattern of *Tc ser,* a gene involved in the formation of segments in appendages, was investigated in *Tc Dfd* knock down embryos.

Therefore in order to further study the effects of *Tc Dfd* knock down on leg appendage segmentation, a marker of leg segmentation, *Tc ser,* was studied in combination with the PD domain genes *Tc Dll* and *Tc dac* to use as markers of segment identity. By determining the expression of *Tc ser* relative to the domains of *Tc Dll* and *Tc dac*, it is possible to determine if maxilla appendage segments have been lost or not, or if they have significantly altered development from wild type maxilla.

Three or four rings of *Tc ser* expression are present in the maxilla of *Tc Dfd* knock down embryos (fig.5.6B,D-J). But, due to the morphology of the mutant appendage, it is difficult to identify which ring of *Tc ser* relates to which segment. The affected maxilla lacks endites and the affected palp is larger and more continuous with the protopodite, which means that there is little morphological frame of reference to identify each *Tc ser* ring of expression. Consideration of the conclusions from the cuticle preparation maxilla phenotype of Dfd^{RNAi} larvae support the first two rings of *Tc ser* expression represent the subcoxa and coxa domains of *Tc ser* expression.

To facilitate the identification of the rings of *Tc ser* expression, Double *in situ* hybridisations were performed with *Tc ser* and the leg gap genes *Tc Dac* and *Tc Dll*. By comparing the relative positions of both of these genes relative to *Tc ser* expression in wild type embryos it is possible to identify the rings of *Tc ser* expression in the mutant maxilla of *Tc Dfd* knock down embryos.

There are clearly three domains of *Tc ser* expression in the affected maxilla (fig.5.6B). Comparison of expression to PD domain gene expression confirms that the first three proximal *Tc ser* domains relate to their wild type counterparts and are therefore not regulated by *Tc Dfd*. The proximal-most ring of expression is the subcoxal-1 *Tc ser* domain, and the second *Tc ser* ring of expression is the coxal-2 ring domain. The third ring of expression relates to the trochanteral-3 ring domain of *Tc ser* which is expressed at the base of the palp (fig.5.6).

The evidence for this conclusion is based upon the expression of *Tc dac* and *Tc Dll* relative to *Tc ser* ring domains in a manner which is reminiscent of wild type appendages. The proximal domain of *Tc dac* is expressed between the subcoxal-1 and coxal-2 ring domains in the affected appendage (star in fig.5.6D,G,H). The distal domain of *Tc dac* is expressed abutting the third ring domain of *Tc ser* (arrow in fig.5.6DG,H). *Tc Dll* is expressed in the distal-most region of the affected palp up to and including the trochanteral-3 ring domain of *Tc ser* (fig.5.6I). A schematic of these

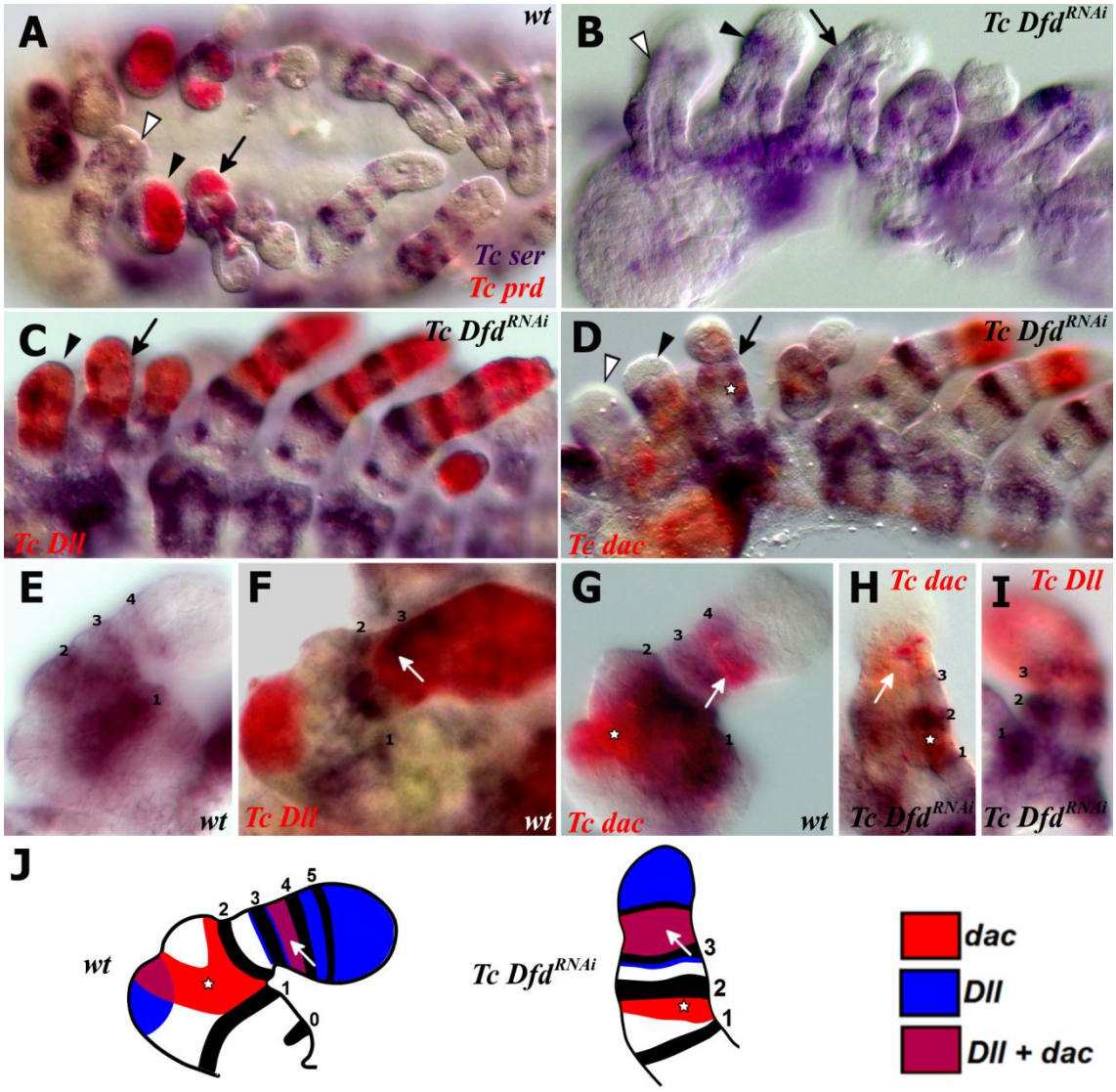

Fig.5.6. The coxal-2, subcoxal-1 and trochanteral-3 domains of *Tc ser* are regulated independently of *Tc Dfd* in the maxilla. Comparison of the expression of the PD domain genes relative to each other and to *Tc ser* expression in the *Tc Dfd*^{RNAi} maxilla shows that the first three ring domains of *Tc ser* are present in the affected maxilla. The proximal domain (star) of *Tc dac* is expressed in between the subcoxal-1 and coxal-2 domains of *Tc ser*. *Tc Dll* is co-expressed with the trochanteral-3 domain of *Tc ser*. The distal domain of *Tc dac* (white arrow) is expressed distally adjacent to the trochanteral-3 *Tc ser* domain. Therefore, *Tc Dfd* is not required for activation of these three proximal rings of *Tc ser* expression. Gene expression was detected by *in situ* hybridisation. (A) Expression of *Tc ser* (blue) and *Tc prd* (red) in a wild type germ band retracting stage embryo. *Tc prd* is expressed in the mandibular, maxillary and labial endites. (B) Expression of *Tc ser* (blue) in a *Tc Dfd*^{RNAi} embryo. Lateral view. The mandible is transformed to antennal identity, the maxilla has three visible *Tc ser* ring domains. (C) Expression of *Tc Dll* (red) and *Tc prd* (blue) in a *Tc Dfd*^{RNAi} embryo. Lateral view. *Tc Dll* expression abuts the coxal-2 *Tc ser* domain in the affected maxilla. (D) Expression of *Tc dac* (red) and *Tc ser* (blue) in a *Tc Dfd*^{RNAi} embryo. Lateral view. Both the proximal and distal domain of *Tc dac* is present in the affected maxilla. (E) Expression of *Tc ser* in a wild type maxilla. (F) Expression of *Tc ser* (blue) *Tc Dll* (red) in a wild type maxilla. *Tc Dll* expression overlaps with the trochanteral-3 domain of *Tc ser* expression. (G) Expression of *Tc ser* (blue) *Tc dac* (red) in a wild type maxilla. (H) Expression of *Tc ser* (blue) *Tc dac* (red) in a maxilla from a *Tc Dfd*^{RNAi} embryo. (I) Expression of *Tc ser* (blue) *Tc Dll* (red) in a maxilla from a *Tc Dfd*^{RNAi} embryo. (J) Schematic of the expression of *Tc ser, Tc dac* and *Tc Dll* in a wild type and *Tc Dfd*^{RNAi} maxilla. Mandibular (arrowhead), maxillary (arrow) and antennal (white arrowhead) segment appendages are indicated. Proximal domain (star) and distal (white arrow) domains of *Tc dac* are indicated.

expression domains in both the maxilla of normal and *Tc Dfd* knock down embryos is shown in fig.5.6J.

In *Tc Dfd*^{RNAi} embryos therefore there is no effect on the expression of the proximal ring domains of *Tc ser* in the maxilla. The first, second and third rings of *Tc ser* expression are present and the expression domains of *Tc Dac* and *Tc Dll* relative to these domains confirm them to be the subcoxa (1), coxa (2) domains of the protopodite and the third (3) *Tc ser* ring domain at the base of the palp.

Ring domains of *Tc ser* expression in the transformed mandible are changed to a pattern typical of the antenna which is consistent with the homeotic transformation of mandible to antenna identity (fig.5.7). *Tc dac* is expressed in between the first and second rings of *Tc ser* expression in the transformed mandibular appendage (fig.5.7D). *Tc Dll* is expressed in the distal part of the transformed mandible to the first proximal ring domain of *Tc ser* (fig.5.7E). Expression of the PD domain genes *Tc Dll* and *Tc dac* in the transformed mandibular appendage is therefore similar to that of the antenna, confirming homeotic transformation of these appendages (shown schematically in fig.5.7F).

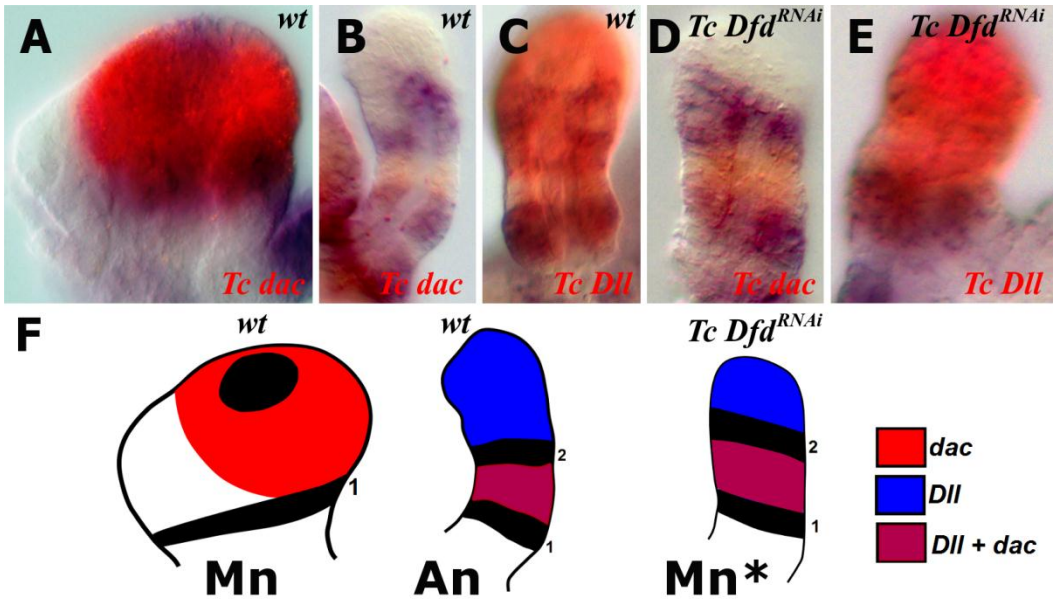

Fig.5.7. The mandible is transformed into antennal identity in *Tc Dfd* embryos. Transformation of the mandible to antennal identity is confirmed by the expression of the PD domain genes *Tc dac* and *Tc Dll* and the segmentation marker *Tc ser.* Expression of these three genes in the transformed mandible replicate the antennal expression domains of these genes. (A) *Tc dac* and *Tc ser* expression in a wildtype mandible. (B-C) Wild type antennae at a slightly later stage than C-E. First antennal domain of *Tc ser* is in the Antennifer segment. The second antennal domain of *Tc ser* is in the scapus. (B) *Tc dac* and *Tc ser* expression in a wildtype antenna. (C) *Tc Dll* and *Tc ser* expression in a wildtype antenna. (D) *Tc dac* and *Tc ser* expression in the transformed mandible of a *Tc Dfd*^{RNAi} embryo. (E) *Tc Dll* and *Tc ser* expression in the transformed mandible of a *Tc Dfd*^{RNAi} embryo. (F) Schematic of the expression patterns of the PD domain genes *Tc dac* and *Tc Dll* and the segmentation marker *Tc ser* in the mandible (Mn), antenna (An) and transformed mandibular appendage (Mn*).

5.3 Discussion

General overview

Knock down of *Tc Dfd* by RNAi shows that *Tc Dfd* is necessary to activate protopodite and endite patterning genes in the maxillary appendages, in addition to repressing antennal development in the mandible as previously described (Brown *et al., 2000)*. Segmentation is unaffected, at least in the proximal part of the affected maxilla as determined by the expression of *Tc ser*. The affected maxilla therefore retains the protopodite. There are significant morphological changes to the protopodite in *Tc Dfd* knock down embryos. The protopodite has lost the endites. The affected maxilla retains the protopodite segments, the subcoxa and the coxa, but has been reduced in size (primarily width). It is also apparent that *Tc Dfd* is necessary to shape the maxilla protopodite. The wild type maxilla protopodite is orientated perpendicular to the palp. In the *Tc Dfd* knock down embryos, the maxilla appendage has lost its characteristic maxilla shape and is more telescopic in appearance.

Tc Dfd is necessary to pattern the endites as shown by study of the expression of *Tc prd* in *Tc Dfd* knock down embryos. *Tc Dfd* activates *Tc prd* expression in the mandibular and maxillary endites. *Tc prd* expression still remains in the labial appendage endites.

Tc Dfd also up-regulates the proximal domain of *Tc dac* in the maxilla. *Tc dac* is upregulated by *Tc Dfd* but not necessarily activated by it. The proximal domain of *Tc dac* expression is weaker in the maxilla and distal expression domain of *Tc dac* is stronger in *Tc Dfd* knock down embryos. As there is still faint expression of the proximal domain of *Tc dac* in *Tc Dfd* knock down embryos, it indicates that *Tc dac* doesn't require *Tc Dfd* for initial activation. It is possible that the RNAi knock down phenotype was not fully penetrant in these experiments and as a result there was only partial knock down of *Tc Dfd* gene function which may reveal itself by partial reduction of *Tc dac* activation. This criticism could also apply to analysis of the lack of an apparent effect on *Tc ser* expression. There was however no posterior collar domain of *Tc cnc* (fig. 3.3B-D) and no obvious partial phenotype like reduced endites, or mandible-antenna hybrid appendages.

The developing limb axis of the maxilla is not controlled by the action of *Tc Dfd.* This conclusion can be made as the maxilla of *Tc Dfd* knock down embryos develops

proximal segments which retain subcoxa, coxa and trochanter identity determined by the expression of the PD domain genes relative to the proximal ring domains of *Tc ser*. Proximal *ser* expression is unaffected in *Tc Dfd* knock down embryos. Using the expression of *Tc Dac* and *Tc Dll* as markers for the subcoxa and coxa demonstrates that the subcoxa and coxa domains of *Tc ser* expression are still present. This situation is unlike that in *Drosophila* where *Dfd* has been shown to regulate regulation of *Tc ser* transcription in the gnathal lobes. Although it is possible that *Tc Dfd* is required for patterning the segments of the palp and for patterning the palp.

The morphology of the maxilla palp is affected in *Tc Dfd* knock down embryos, although the precise details were not deciphered. The base of the transformed maxillary palp is larger in developing embryos, lacking the characteristic inverse taper as the diameter of the maxilla palp decreases proximally to a narrow base that attaches to the maxilla protopodite. In first instar larvae, the maxilla is smaller in Dfd^{RNAi} larvae than wild type larvae. There also appears to be a reduced number of segments in the larval palp. However, it was difficult to detect expression domains in the maxillary palp in general, and it was also difficult to detect segmentation in the larval cuticle preparations of both wild type and knock down larvae so more research would have to be performed on the role of *Tc Dfd* in the palp to be more certain.

The identity of the proximal maxilla appendage.

It has been shown previously by Brown *et al.* that the Hox gene necessary for proximal labial identity the *Tribolium* homologue of *Scr, Cephalothorax* (*Cx),* is not expressed in the maxillary segment of *Tc Dfd* mutant embryos (Brown et al., 2000). This result shows that the affected proximal maxilla is not of labial identity, despite resemblance of the larval maxilla to half the labial appendage (which is made up of two separate appendages, left and right, which later fuse centrally during embryogenesis). It has been shown in *Drosophila* that *Dfd* activates *pb.* Expression of *mxp,* the *Tribolium* orthologue of *pb,* has not been tested[14] in *Tc Dfd* knock down larvae or mutants. Therefore in order to confirm that *mxp* is still expressed in the maxillary appendage in *Tc Dfd* knock down embryos expression was detected by *in situ* hybridisation.

[14] At least *mxp* expression has not been studied in *Tc Dfd* mutants in the published literature.

Without a Hox gene present in the base of the maxillary appendage in *Tc Dfd*^{RNAi} embryos, it is not immediately obvious what the identity of the base of the transformed maxillary appendage will be. It is likely that the presence of *mxp* will repress antennal development in the maxilla and activate the post-antennal P/D axis genetic pathway.

The affected maxillary protopodite could have a generic non-antennal proximal identity lacking the specific input of Hox genes. The resemblance of the *Tc Dfd*^{RNAi} maxilla to half of the labial appendage may represent the sharing of default P/D axis patterning mechanism at the base of the maxillary appendage as there is no Hox gene expressed at the base of the appendage. The maxilla is patterned in an additive fashion by two Hox genes, *Tc Dfd* and *mxp*. Expression of a Hox gene is required in the ectoderm for appendage specification. Without a Hox gene present, the appendage primordia patterns antennal appendage by default. Considering that in *Tc Dfd*^{RNAi} embryos, there is still a Hox gene present that can repress antennal identity, *mxp,* means that it is unlikely that the base of the maxilla has been transformed to antennal identity.

It is possible that another PD domain gene is affected by loss of *Tc Dfd* function. *Tc hth* is expressed in the mandible and the protopodite of all post-antennal appendages. In *Drosophila,* the proximal coxal ring of *ser* expression is activated by *hth* (Rauskolb, 2001). Considering that the proximal domains of *Tc ser* are apparently unaffected by *Tc Dfd* knock down in *Tribolium*, if *Tc hth* has a conserved function to activate proximal domains of *Tc ser,* it is reasonable to assume that *Tc hth* will not be affected by *Tc Dfd* Knock down. This will have to be tested in order to prove this hypothesis.

Comparison of *Tc Dfd* function with *Dfd* in *Drosophila*

The function of *Tc Dfd* in *Tribolium* is very similar to the function of *Dfd* in *Drosophila.* Both genes pattern structures derived from the proximal part of the maxillary gnathal lobe. In *Drosophila, Dfd* is necessary to pattern mandible and proximal maxillary gnathal lobe derived structures such as the cirri and ventral organ of the maxillary gnathal lobe which are hypothesized to be homologous to the endites of the maxilla (Jurgens et al., 1986). *Tc Dfd* is necessary to pattern the maxilla protopodite, especially the endites, and the mandible. There are also similar genetic

interactions present in both species. In both *Drosophila* and *Tribolium, Dfd* regulates the proximal domain of *Dll* and regulates *prd* in the maxillary gnathal lobe. There are however several differences between *Dfd* in *Drosophila* and *Tribolium.* In *Tribolium, Tc Dfd* regulates *Tc dac* and activates *Tc cnc*. In *Drosophila, Dfd* has been shown to regulate *ser* and *mxp* which does not occur in *Tribolium* as is shown above.

Dfd is necessary to activate three genes that are important for patterning the proximal part of the maxillary gnathal lobe in *Drosophila*. *Dfd* activates the proximal domain of *Dll* by a maxillary-specific enhancer (called ETD6) and is required for the formation of proximal maxillary lobe derived structures, the cirri (O'Hara et al., 1993). *Dfd* activates the late expression domain of *prd* in the proximal region of the gnathal lobes (Gutjahr et al., 1993; Li et al., 1999). *prd* is necessary for proximal maxillary lobe derived structures (such as the cirri and the ventral organ) (Vanario-Alonso et al., 1995). *ser* is also a target of *Dfd* in the mandibular and maxillary lobes. *ser* is required for normal mouth hook development (Wiellette and McGinnis, 1999).

In *Drosophila ser* is regulated by *Dfd. Tc ser* does not appear to be regulated by *Tc Dfd* as *Tc ser* ring domains are present in knock down embryos. However, it was not possible to determine whether there is any effect on more distal domains of *Tc ser*¸ primarily because the staining of *Tc ser* is weak in the distal part of the maxilla. Comparison of the co-expression of PD domain genes with *Tc ser* shows that the three proximal domains of *Tc ser* in the affected maxilla are still present (fig.5.6.)

Another significant difference relates to the regulation of *dac.* In *Tribolium, Tc Dfd* regulates the proximal domain of *Tc dac,* whereas in *Drosophila dac* is not expressed in the developing gnathal lobes of *Drosophila* and therefore is not be regulated by *Tc Dfd* (Tomancak et al., 2002; Tomancak et al., 2007).

In *Drosophila, pb* is activated by *Dfd* in the mandibular and maxillary segment (Rusch and Kaufman, 2000). In *Tribolium, mxp* is not activated by *Tc Dfd.* Knock down of *Tc Dfd* did not result in any decrease in *mxp* in the maxillary segment. In the mandibular segment *mxp* is expressed in the posterior of the transformed mandibular appendage.

As shown in chapter four, *Tc Dfd* activates the posterior collar domain of *Tc cnc* in the mandibular segment. This is in contrast to the *Drosophila,* where it has been shown that *Tc cnc* is activated by gap genes and pair rule genes and is activated independently of Hox genes (Mohler, 1993).

Experiments on other Mandibulates

Tc Dfd is important for patterning the protopodite of the maxilla in *Tribolium*. In the maxilla protopodite, *Tc Dfd* regulates two genes, *Tc dac* and *Tc prd* which also have pronounced expression domains in the mandibular endite. Therefore, it is likely that *Tc Dfd* will also activate *Tc dac* and *Tc prd* in a similar manner in the mandible. It is anticipated that the mandibular expression domains of these two genes are modified by *Tc cnc* after their initial activation by *Tc Dfd*, as part of the genetic program that specifies mandibular identity.

The conservation of the proximal patterning function of *Dfd* in *Tribolium* and *Drosophila* and the similarity of expression of *Dfd* orthologues in other mandibulates suggests that the proximal maxilla patterning function of *Dfd* may be conserved in other mandibulate organisms. If the maxilla-to-mandible differentiating function of *cnc* is found to be conserved in mandibulates, as comparative expression data suggest, then an understanding of how the mandible evolved from a maxilla-like precursor from a molecular developmental perspective can be obtained by comparing the development of both the mandible and the maxilla in diverse mandibulates.

In chapter seven, I will investigate expression the findings from the previous three chapters regarding the role that *Tc cnc* has been demonstrated to have in *Tribolium*. I will outline a hypothesis of how *Tc cnc* functions to pattern the mandibular appendage of *Tribolium* together with the protopodite patterning function of *Tc Dfd* as outlined in this chapter. I will then conclude as to what the ancestral patterning genes of the gnathocephalon in Mandibulata may have been based upon by comparing the expression of the genes necessary to pattern the gnathocephalon of *Tribolium* to the expression of these genes in other mandibulates, with particular focus on the evolution of the mandibular endite.

Chapter 6:
Investigating the role of mandible patterning genes in non-mandibulate arthropods

6.1 Introduction

In order to study how conserved mandible patterning genes evolved in the ancestor to mandibulates it is necessary to study these genes in outgroups of mandibulates to try to determine their ancestral function. *cnc* and *Dfd* are two good candidate genes to have this role as shown in chapters four and five, therefore an investigation into the expression of the homologue of *cnc* in a non-mandibulate was undertaken. It was originally planned that the function of the spider *cnc* homologue and the two spider homologues of *Dfd* would be investigated. However, gene knockdown by parental RNAi was unsuccessful in positive controls and was therefore not continued.

It is the contention of this thesis that the arthropod mandible evolved from a leg through a maxilla-like precursor to form the mandible. Evidence that the mandible has evolved from a leg is evident from comparisons of the mandibular segment to the homologous segment in non-mandibulate arthropods and stem lineage arthropods (Waloszek et al., 2007; Chen, 2009). In all non-mandibulates such as chelicerates, there is a locomotory leg present on the homologous segment to the mandibular segment. Non-mandibulate Cambrian arthropods such as the Lamellipedia (which includes trilobites), megacheirans and other stem group arthropods are characterised by numerous undifferentiated serially homologous biramous leg appendages, one of which is serially homologous to the arthropod mandible (Boxshall, 2004).

Evolution of the mandible from a maxilla-like precursor is evident in the structure of maxilla-like appendages present on numerous stem lineage crustaceamorph fossils present on the second post-antennal segment (Muller and Waloszek, 1986; Siveter et al., 2001; Waloszek et al., 2007). The Hox gene *Dfd* is expressed in the mandibular segment and patterns the mandible in combination with

cnc in *Tribolium* and *Drosophila.* Without *cnc, Dfd* patterns maxillary structures. This genetic interaction may represent an important part of the evolutionary history of the mandible, with the original function of *Dfd* involved in a maxilla or biramous limb appendage specifying function and *cnc* recruited to differentiate the mandible protopodite from the maxilla or biramous limb protopodite.

cnc is a basic Leucine zipper (bZIP) member of which examples are present in the majority if not all Bilaterians (Mohler et al., 1991; Grimberg et al., 2011). bZIP proteins are characterized by a conserved domain that includes a basic DNA binding domain upstream of a Leucine zipper that consists of several heptad repeats. The repeating residue in the heptad repeat is typically a Leucine, or another hydrophobic residue such as Isoleucine or Valine. These hydrophobic residues are able to 'interlock' with other hydrophobic residues present on other Leucine zipper proteins to form dimers. Numerous CNC family members are only capable of forming heterodimers due to the presence of charged amino acid residues in the Leucine zipper domain that prevent homodimerization (Mohler et al., 1991). *cnc* present in *Drosophila* is one such example; it is unable to form homodimers and forms heterodimers with a small Maff protein MafS (Veraksa et al., 2000). The *Caenorhabditis elegans* CNC family member *Skn-1* has lost the Leucine zipper region of the bZIP domain and consequently is able to function as a monomer (Walker et al., 2000).

Alignment of CNC family representatives from metazoans has shown that there was one ancestral CNC family gene that has been duplicated more than once in the lineage leading to the chordates (Grimberg et al., 2011). There is only one CNC family member present in arthropod taxa (Mohler et al., 1991) and one present in the nematode *C. elegans, Skn-1* (Bowerman et al., 1992; Walker et al., 2000). Four homologous CNC genes are located 3' to the four Hox clusters of vertebrates: *NF-E2 (Andrews et al., 1993), Nrf1* also known as *LCR-F1* in mice (Chan et al., 1993; Caterina et al., 1994; Chan et al., 1996; Farmer et al., 1997)*, Nrf2* or *ECH* (Moi et al., 1994; Itoh et al., 1995)*,* and *Nrf3* (Kobayashi et al., 1999; Etchevers, 2005). Functions of CNC family members are diverse, but often include conserved functions such as the specification of midgut and pharyngeal tissue, roles in hematopoiesis, and xenobiotic and oxidative stress responses in addition to the developmental functions present in arthropods (Blackwell et al., 1994; Itoh et al., 1997; Alam et al., 1999; Wild et al., 1999; Walker et al., 2000; Grimberg et al., 2011). Despite the proximity of CNC family

members to Hox gene clusters, no interaction between CNC homologues and Hox genes has been observed outside of arthropods (Etchevers, 2005).

In order to investigate the primitive functions of the mandibular patterning genes *cnc* and *Dfd,* it is necessary to study their expression and function in groups outside of the mandibulates. The function, and indeed expression of *cnc* is unknown in non-mandibulate arthropods. Prior to this current investigation, it was not known whether there was a *cnc* orthologue present in any non-mandibulate arthropod taxa. The only extant non-mandibulate arthropod group is the Chelicerata, which includes the arachnids (spiders, scorpions, ticks, mites and harvestmen) and xiphosurans (horseshoe crabs). Less closely related taxa (present in Panarthropoda) include the Onychophora (velved worms) and the Tardigrada (Brusca and Brusca, 2003; Budd and Telford, 2009).

The homologous segment to the mandibular segment in chelicerates is the first walking leg segment. The strongest evidence for this comes from examination of the anterior boundary of Hox gene expression. Comparison of the anterior limit of Hox gene expression in mandibulates reveals that the expression domains are conserved. For example, the anterior limit of *labial* and *proboscipedia* is present in the first post-antennal segment which is the second antennal segment of crustaceans and the intercalary segment of myriapods and hexapods. The anterior boundary of *Dfd* is present in the second post-antennal segment which is the mandibular segment of all mandibulates (Hughes and Kaufman, 2002a; Scholtz and Edgecombe, 2006).

Comparison of anterior Hox gene expression domains of *lab*, *pb, Hox3, Dfd* and *scr* in the spiders *Cupiennius salei, Achaearanea, Steatoda triangulosa* and the orbatid mite *Archegozetes longisetosus* enabled segmental homologies to be inferred between the chelicerates and the mandibulates. The chelicerae bearing segment is homologous to the antennal segment, as there are no Hox genes expressed in these segments. The first post-cheliceral segment, the pedipalp segment, is homologous to the second antenna/intercalary segment (marked by the anterior boundary of *lab* and *pb).* The second post-cheliceral/antennal segment, the first leg segment, is homologous to the mandibular segment and is marked by the anterior boundary of *Dfd* expression (Damen et al., 1998; Telford and Thomas, 1998b; Abzhanov and Kaufman, 1999a; Schwager et al., 2007). The segmental expression of the full complement of Hox genes of the spider *Cupiennius salei* is shown in fig.6.1.

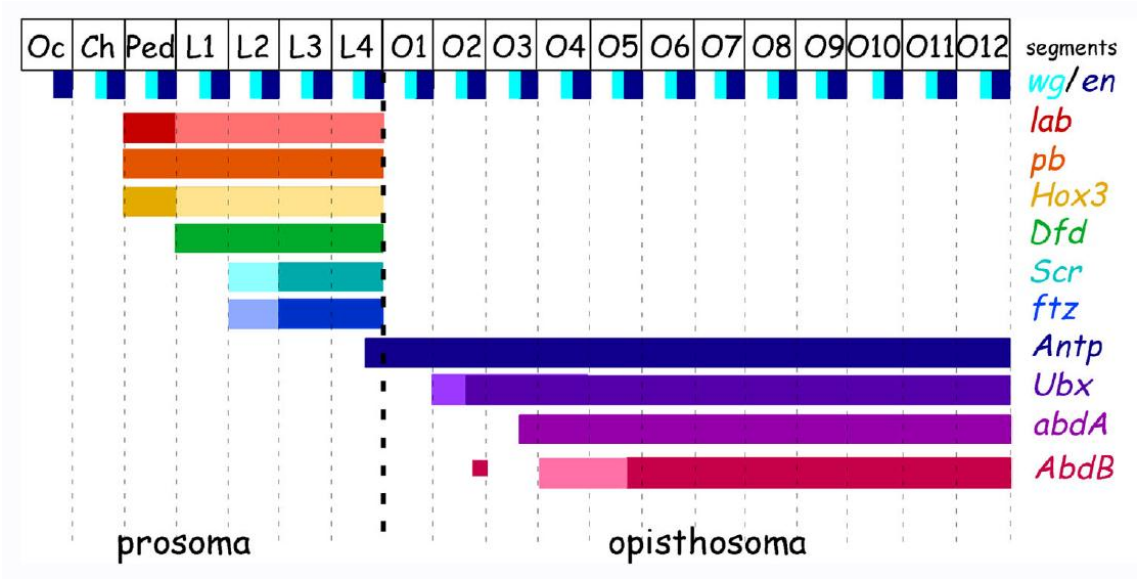

Fig.6.1. Segmental expression patterns of the ten Hox genes of *Cupiennius salei.* Anterior is to the left. Segments are in anterior to posterior order: ocular (Oc), chelicerae (Ch), pedipalps (Ped) and four Leg segments (L1-L4) opisthosomal segments (O). The Prosoma/opisthosoma boundary is indicated with a dotted line. *wg* and *en* expression marks the posterior of each segment. There are duplications of Hox genes *Dfd, Scr* and *Ubx.* The homologous segment to the mandibular segment is the L1 segment. The L1 segment has *lab, pb, Hox3* and *Dfd* expression. The anterior expression boundary of *Dfd,* (which may relate to its function) is expressed in the L1. Note the posterior boundary of the expression domains of anterior Hox genes *lab, pb, Hox3, Dfd, Scr* and *ftz* extend to the most posterior prosomal segment the L4. The expression of the posterior Hox genes *Antp, Ubx, abdA, AbdB* extends to the posterior opisthosomal segment (O12). Figure from (Schwager *et al.*, 2007).

Outside of Chelicerata and Mandibulata, expression of *lab, pb* and *Dfd* in a pycnogonid *Endeis spinosa* homologises the mandibular segment to the third pair of larval appendages (Jager et al., 2006). Study of the expression of Hox genes in an onychophoran *Euperipatoides* homologises the mandibular segment to the first walking leg of this species (Eriksson et al., 2010).

The legs of chelicerates present on the L1 to L4 segments are nearly identical to one another. The chelicerate L1 to L4 segments are homologous to the mandible, maxilla, labial and first thoracic segments of insects, respectively, which are clearly differentiated from each other. In *Tribolium,* differentiation of these four segments is accomplished by the Hox genes *Tc Dfd, Cx, mxp* and *prothorax-less* (*ptl*) (the *Tribolium* orthologue of *antennapedia*) (Brown et al., 2000; Shippy et al., 2000b; Curtis et al., 2001; DeCamillis et al., 2001; Brown et al., 2002a). There is no Hox gene that differentiates the mandibular segment from the maxillary segment. Instead, this is accomplished by the bZIP gene *cnc* (as shown in chapter four). In chelicerates, there has been no functional study of the function of anterior Hox genes. To date only one Hox gene, *antp*, has been studied functionally. *antp* represses appendage formation in

the first opisthosomal segment which is the anterior-most segment where *antp* is expressed (Prpic, personal communication). There have been duplications of some Hox genes in some chelicerates, such as *Dfd, Ubx,* and *Scr* (Abzhanov et al., 1999; Schwager et al., 2007).

There are numerous Hox gene candidates for differentiating the second and third post-cheliceral appendages of chelicerates (the first and second leg appendages) based on the anterior limit of their expression domains. The anterior boundary of the two homologues of *Dfd* are expressed in the L1 segment and the anterior boundary of two hox genes *Scr* and *ftz* is present in the third post-antennal segment.

Considering that the first leg segment of chelicerates is not differentiated in any obvious morphological manner from the second leg segment, and there are numerous Hox genes that could potentially perform this role, it seems that there would be no requirement for *cnc* to differentiate this segment from the second leg segment. It would therefore be of interest to study *cnc* in a chelicerate to find out exactly what role, if any, there is of the posterior coller domain present in the mandibular segment of mandibulate arthropods which is homologous to the first leg segment of chelicerates. If there was no posterior domain of *cnc* present in the chelicerates, it would provide evidence that this domain has been acquired in the ancestor to mandibulates.

In *Tribolium, Tc cnc* differentiates the mandible from a maxilla and represses the Hox gene *Tc Dfd.* In all studied chelicerates, *Dfd* is expressed in a broad domain from the first leg segment, the homologous segment to the mandibular segment, to the fourth leg segment. It would also be interesting to study the interaction between spider homologues of *cnc* and *Dfd*, to determine whether there is any genetic interaction between *cnc* and *Dfd* (or any other Hox gene)*,* such as present in *Tribolium* or whether this interaction evolved in the lineage leading to the mandibulates.

All non-mandibulate groups, the Chelicerata, Onychophora and Tardigrada are potentially informative of the ancestral function of *cnc* in the ancestor to mandibulate arthropods*.* However, onychophorans and tardigrades are harder to study (if at all in the case of the tardigrades), with no means of attempting functional genetic analysis and are less closely related to mandibulate arthropods. Chelicerates, on the other hand, are the sister group of the mandibulate arthropods and have the potential for functional genetic analyses. Developmental data comparing the development of the

homologous segment of the second post-cheliceral/antennal appendage could also provide evidence to favour either of the competing phylogenetic hypotheses of the Mandibulata and the Myriochelata.

I therefore chose a suitable chelicerate to study the function of a non-mandibulate orthologue of *cnc*, the house spider *Achaearanea tepidariorum*[15]. The embryonic development of *Achaearanea* has been studied in the laboratory for several years, and so techniques such as *in situ* hybridization and RNAi are now routinely used to investigate its development (McGregor et al., 2008).[16] The expression of the genes *At Dll, At en* and *At Dfd-1* were studied both as controls for *in situ* hybridization experiments and to study the developing spider embryo.

[15] *Achaearanea tepidariorum* has recently been reclassified as *Parasteatoda tepidariorum.* As there is an inherent inertia in the literature to reclassify familiar terminology and the majority of studies still refer to *Achaearanea* and not *Parasteatoda,* this convention will also be followed here.

[16] Although, RNAi is somewhat problematic and feasibly limited to studying the function of early expressed developmental genes.

6.2 Results

Cloning the *At cnc* orthologue

In order to clone the homologue of *cnc* in *Achaearanea,* degenerate primers were designed against conserved regions of the *cnc* gene locus. Nested PCR using degenerate PCRs was performed to increase both specificity and yield of the amplified product. A PCR reaction was performed using the degenerate primer sequences based on the amino acid sequence SRDEKRA and KRKLDQI for the forward and reverse primers respectively. Amplification of the product of the first PCR of outer primer pairs was performed with nested degenerate primers based on the amino acid sequence PIDEFNE and KVAAQN for the forward and reverse primers respectively. The nested PCR reaction produced a 110bp product of which 71bp was non-primer based amplified sequence that was aligned to other arthropod homologues of *cnc* (see fig.6.2).

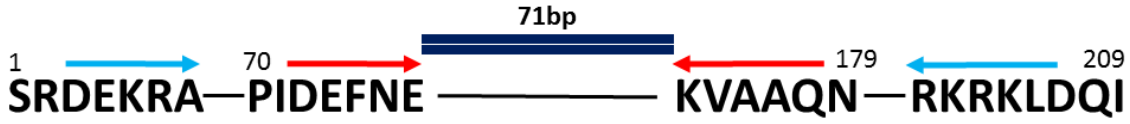

Fig.6.2. Degenerate primers used to amplify homologue of *cnc* in *Achaearanea.* Nested PCR was performed with two pairs of degenerate primers: forward outer primer SRDEKRA and reverse outer primer RKRKLDQI were used to amplify a DNA template for the nested PCR reaction. The forward nested primer PIDEFNE and the reverse nested primer KVAAQN were used to amplify a 110 bp product of which 71bp did not include the primer sequence (shown as two dark blue lines). Outer primers are indicated with blue arrows, nested primers are indicated with red arrows. The numbers indicate the nucleotide position of the 5'end of the primer from the position of the first forward primer.

The alignment of this 71bp sequence confirmed the identity of this gene fragment as a *cnc* related gene. The protein alignment of this region is shown in fig.6.3. This sequence of *At cnc* DNA was extended by rapid amplification of cDNA ends (RACE). Two sequences were obtained from RACE, a 596bp sequence from the combined products of 3' and 5' RACE cloning and an additional 714bp sequence from 3'RACE cloning of the 596bp product. The combined total unique sequence of *At cnc* obtained was 1207bp. Both sequences were transcribed to synthesize two labelled anti-sense RNA probes to detect *At cnc* by in *in situ* hybridization. The protein alignment of the bZIP domain of *At cnc* to other CNC family members and bZIP family of transcription factors is shown in fig.6.4. *At cnc* possesses the basic DNA binding

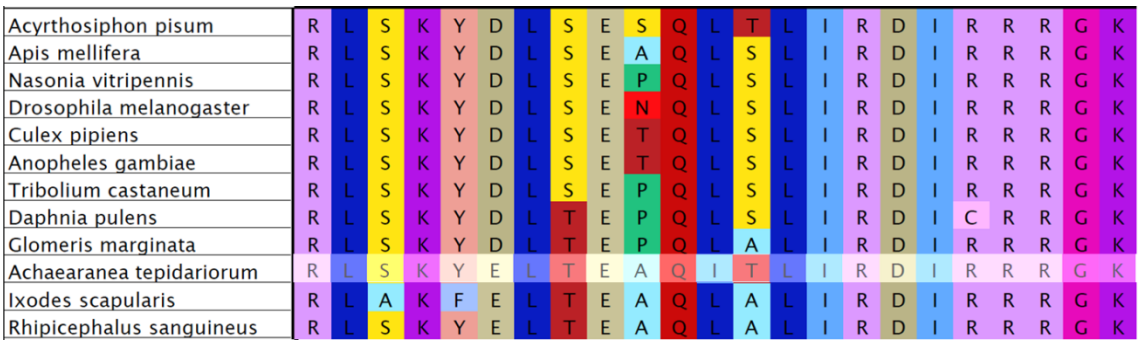

Fig.6.3. Alignment of translated 71bp sequence obtained by degenerate PCR with cnc orthologues from other arthropod taxa confirms identity as a CNC family member in *Achaearanea*. Figure shows a 23 amino acid alignment with half of the basic region of the bZIP domain present at the C-terminal end of the alignment. The *At cnc* sequence is more similar to other chelicerates *Ixodes* and *Rhipicephalus* than other arthropods.

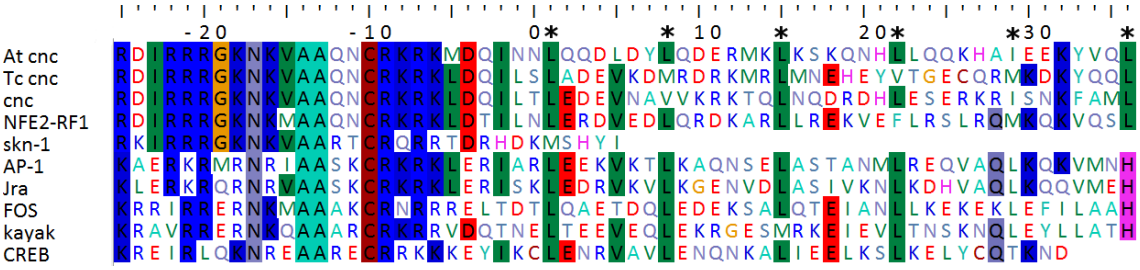

Fig.6.4. Alignment of the bZIP domain of *At cnc* with other CNC and bZIP family members confirms that *At cnc* is a homologue of *cnc*. The basic region, the DNA binding domain, of the bZIP domain is between positions -25 to -3 and consists of numerous basic residues. The Leucine zipper consists of heptad repeats, every seventh amino acid is a Leucine or hydrophobic residue. The leucine zipper positions are indicated with asterisks present at positions 1, 8, 15, 22, 29 and 36. CNC family members are *Tc cnc* from *Tribolium, cnc* from *Drosophila, NFE2-RF1 (Nrf1)* from *Homo sapiens* and Skn-1 from *C. elegans.* bZIP family members include AP-1 (also known as JUN) from *Homo sapiens,* Jun related antigen from *Drosophila melanogaster,* FOS from *H. sapiens, Kayak* (synonymous with Fos related antigen) from *Drosophila,* and cAMP response element binding protein (*CREB*) from *Drosophila. At cnc* is clearly more similar to other CNC family members than other bZIP family members. CNC family members have 6 heptad repeats with some substitutions of alternative amino acids in some of the leucine zipper postions. Charged residues present in some postions in the leucine zipper region can prevent homodimerization. *Skn-1* has lost its Leucine zipper domain but retains the basic region which is highly similar to other CNC family members. Amino acid residues that are shared by 50% or more taxa are highlighted.

domain and the Leucine zipper that is clearly more similar to the CNC family members than the remaining bZIP family of transcription factors.

At cnc transcripts were detected by RT-PCR at all studied stages of embryogenesis from very early blastoderm stages through to late stages prior to hatching of spiderling larval instars.

Control *in situ* hybridizations with the genes *At en, At Dll, At Dfd-1*

In order to optimize the *in situ* hybridization protocol and study the developing embryo of *Achaearanea, in situ* hybridizations were performed with the homologues of the genes engrailed (*At en*), Distal-less (*At Dll*), and to one of the spider *Dfd* homologues (*At Dfd-1*). Sequences for these genes were downloaded from Genbank (Benson et al., 2005). The expression of these genes is shown in fig.6.5., fig.6.6. and fig.6.7. *At en* is expressed in the posterior of each developing segment in the embryo and the posterior of the developing appendages (see fig.6.5.A-D). Additional segmental domains of *At en* expression are added to the posterior of the embryo as the growth zone progressively adds segments to the germ band extending embryo. *At Dll* is expressed in the developing appendages (see fig.6.5. E-H). The appendages in order from anterior to posterior are the chelicerae (Ch), pedipalps (Pp), and the first to the fourth leg appendage segments (L1-L4). The first leg segment, which is homologous to the mandibular segment, is indicated with an arrow.

As the anterior domain of *cnc* in *Tribolium* and *Drosophila* is expressed in the developing labrum, the development of the spider labrum was visualized using *At Dll* as a marker (fig.6.6A-D), and compared to the development of the *Tribolium* labrum which was visualized with *Tc cnc* and *Tc Dll* expression (see fig.6.6F,G). The spider labrum (or *At Dll* expression in the developing labrum) develops relatively late compared to *Tribolium.* The labrum develops as a pair of labral buds in both species which later fuse to form the labrum. *At Dll* is also expressed in a domain around the developing stomodeum (arrowhead in fig.6.5C) which is reminiscent of the *Tc cnc* expression domain around the developing stomodeum in *Tribolium* (arrowhead in fig.6.6F). Conversely, there is no stomodeal domain of *Tc Dll* expression in *Tribolium* (fig.6.6G).

The expression of *At Dfd-1* is shown in fig. 5.6. *At Dfd-1* is expressed in the developing L1 to L4 segment limb buds (fig.6.7C,D). Expression is localized in the tips of these appendages (marked with an arrow in fig.6.7E). Expression of the *Tribolium* homologue of *Dfd* is shown in fig.6.7F. Comparison of the anterior expression domains homologizes the first leg segment to the mandibular segment. The anterior domains of *Dfd* in *Tribolium* and *Achaearanea* is depicted schematically in fig.6.7G. Expression of *At Dfd-1* was visualized by *in situ* hybridization using tyramide signal amplification. This

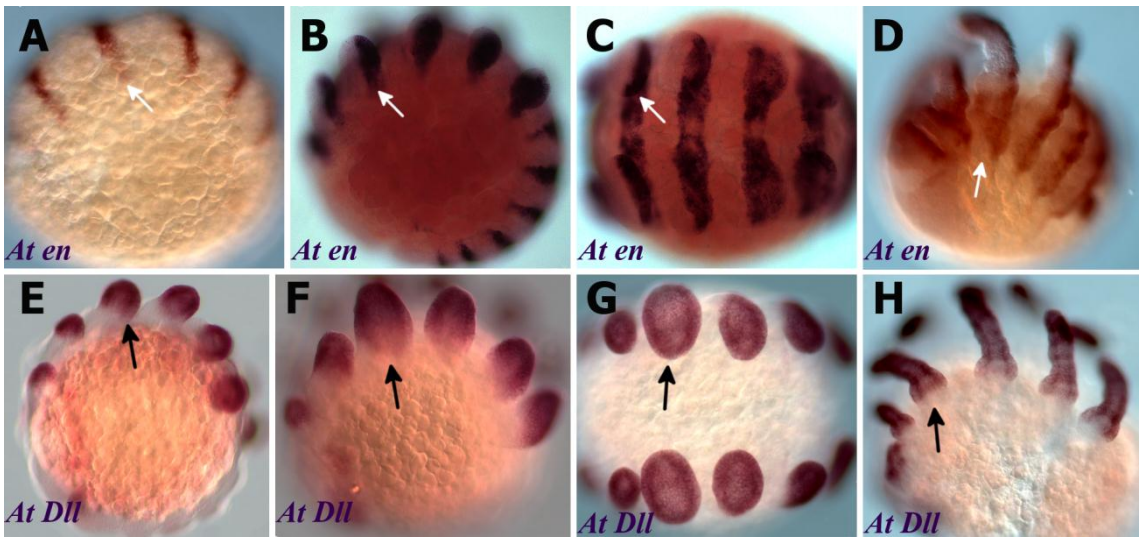

Fig.6.5. Expression of homologues of segment polarity gene *engrailed* and the PD domain gene *Dll* in *Achaearanea* embryos. Gene expression was detected by *in situ* hybridization. The L1 segment is indicated with an arrow. Anterior is to the left. (A-D) Expression of segment polarity gene *At en. At en* is expressed in the posterior of each segment. (A) lateral view of early germ band extending stage. (B) Later germ band extending stage after the limb buds have formed, lateral view. (C) Ventral view of embryo at a similar stage to B. (D) Late stage embryo, lateral view. (E-H) Expression of *At Dll*. *At Dll* is expressed in the distal tips of every appendage. (E) Germ band extending embryo after the limb buds have formed, lateral view. (F) Lateral view of a slightly later stage than E. (G) Ventral view of an embryo at the same stage as F. (H) Later stage germ band retracting embryo.

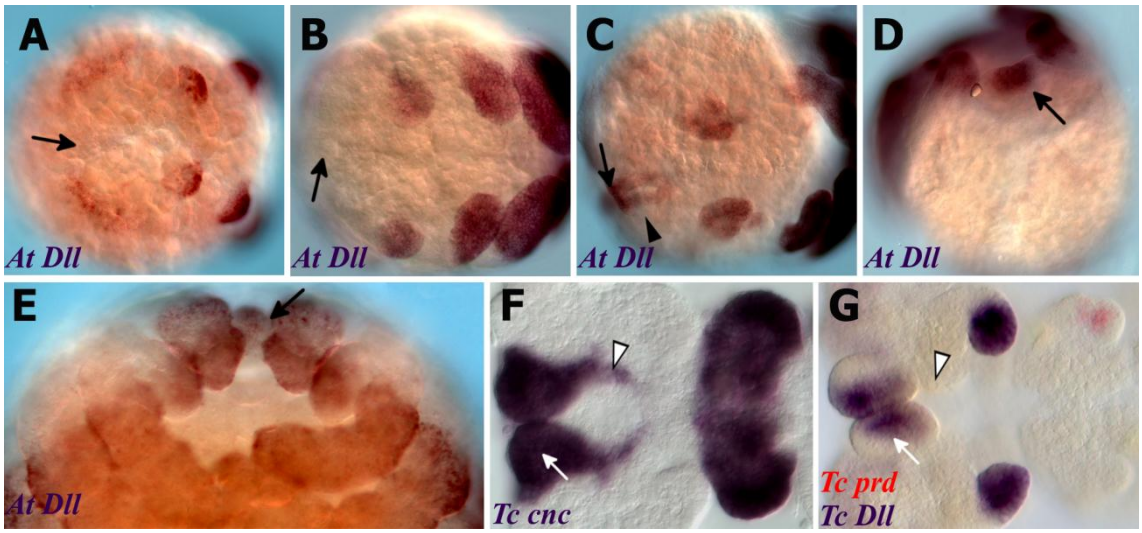

Fig.6.6. Comparison of the labrum of *Achaearanea* to *Tribolium* marked by the expression of *Dll.* Gene expression was detected by *in situ* hybridization. All views are ventral with anterior to the left unless otherwise indicated. (A-E) Expression of *At Dll* in developing spider embryos. (F-G) *Tribolium* embryos. The developing labrum is marked with an arrow. Stomodeal expression domains is marked with arrowhead. (A) Embryo just after the limb buds have formed. There is no expression of *At Dll* in the labrum. (B) Slightly later Germ band extending embryo than A. The stomodeum is visible, but there is no *At Dll* expression. (C) Germ band extending embryo, the stomodeum is surrounded by *At Dll* expression. (D) Germ band extending embryo, the labral buds are beginning to fuse. (E) Late stage embryo during dorsal closure. The labrum is completely fused. Anterior is to the top. (F) Expression of *Tc cnc* in germ band extending *Tribolium* embryo. *cnc* is expressed in an anterior domain in the labrum and a posterior domain in the mandibular segment. Expression is also present around the developing stomodeum in a manner similar to that of *At Dll.* (G) Expression of *Tc Dll* and *Tc prd.* The stomodeal domain of *Dll* present in *Achaearanea* is missing in *Tribolium* (arrowhead).

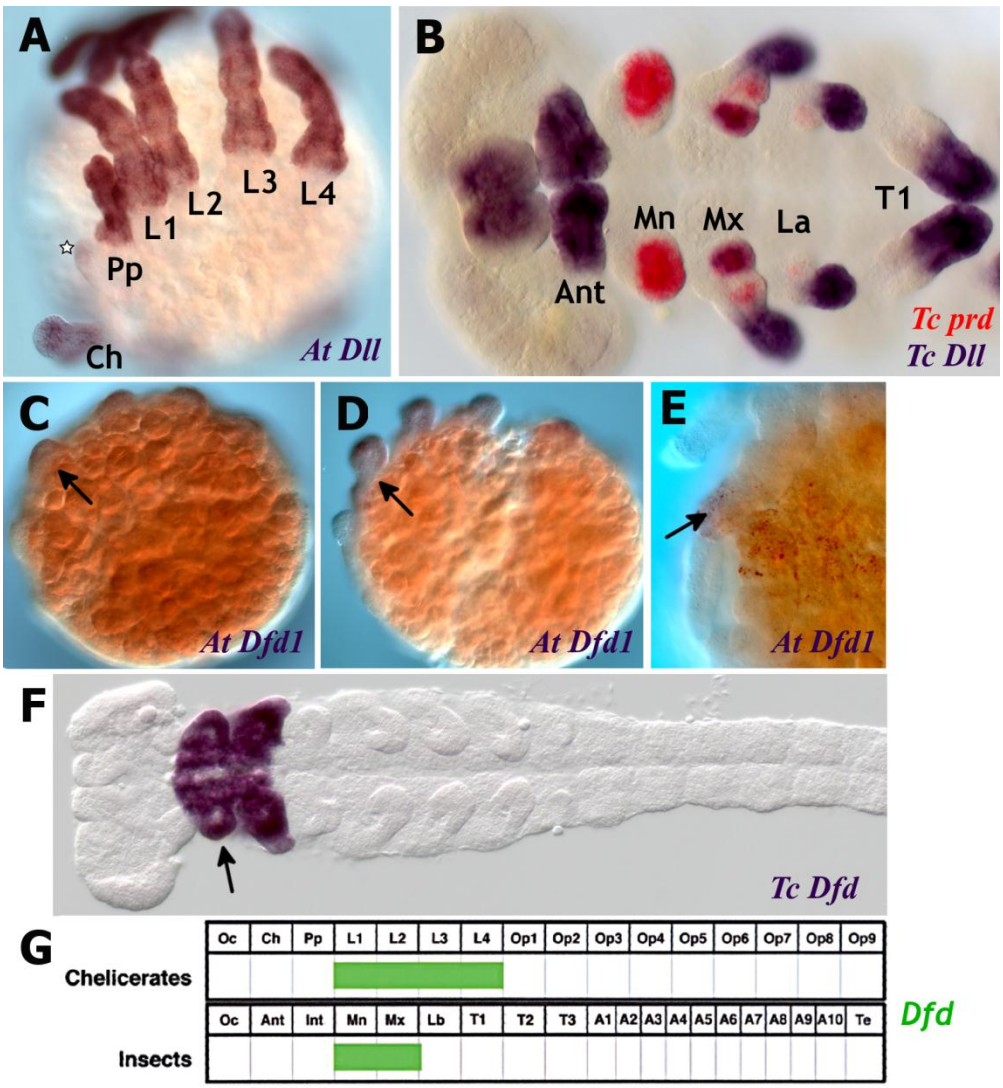

Fig.6.7. Comparison of the anterior expression boundary of *Dfd* homologues in *Achaearanea* and *Tribolium* homologises the mandibular segment to the first leg segment. Gene expression was detected by *in situ* hybridization. (A) Expression of *At Dll* in a late germ band extending embryo. The appendages are clearly marked with *At Dll* expression. There is *At Dll* expression in the endite on the pedipalp (star). (B) Expression of *Tc Dll* and *Tc prd* in *Tribolium* embryo. *Tc Dll* is expressed in the tip of every appendage except the mandible. *Tc Dll* is also expressed in the labrum. *Tc prd* is expressed in the endites of the mandible, maxilla and labial appendages. (C-E) Expression of *At Dfd-1* in *Achaearanea.* Expression of *At Dfd-1* is present in the L1 to L4 segments. (C) Early germ band extending embryo as the limb buds are forming. Arrow marks the anterior boundary of *At Dfd -1* expression. (D) later stage germ band embryo. Arrow marks anterior boundary of *At Dfd-1* expression. (E) *At Dfd-1* expression is present at the tips of appendages (arrow). (F) Expression of *Tc Dfd* in *Tribolium.* The arrow marks the anterior boundary of *Tc Dfd* expression in the mandibular segment. *Tc Dfd* is expressed in the mandibular and maxillary segments. (G) Comparison of the expression domains of *Dfd* between chelicerates and insects represented in the figure by *Achaearanea* and *Tribolium* respectively. Figure is adapted from Hughes and Kaufman (2002a). The segmental abbreviations are as follows: ocular (Oc), chelicerae (Ch), pedipalps (Ped) and four Leg segments (L1-L4) opisthosomal segments (Op), antennal (ant), intercalary (Int), mandibular (Mn), maxillary (Mx), labial (Lb), thoracic (T), abdominal (A)

alternative *in situ* hybridization protocol was tested to try to provide additional sensitivity for detecting transcripts that are expressed at a low level.

Expression of *At cnc* transcripts visualized by *in situ* hybridization

In order to visualize expression of *At cnc,* numerous *in situ* hybridizations were performed. No structure specific signal was detected. Instead, ubiquitous or background staining was visible in all performed *At cnc in situ* hybridization experiments.

Different *in situ* hybridization parameters were used in different experiments in order to optimize the signal of *At cnc* expression. The following parameters were changed in different *in situ* hybridization experiments: i) Length of probe (extending the *At cnc* specific antisense probe sequence from 366bp to 1.2kb). ii) Concentration of probe was tested over a thousand-fold range (0.01µl-10µl labelled RNA probe/100µl hybridization solution). iii) Incubation time of hybridization of the probe to the embryos from overnight to one week. iv) Incubation time of the anti-hapten antibody from overnight to one week.

In addition, the hybridization temperature was tested over a range of 55^{o}C to 65^{o}C. The concentration of formamide present in the hybridization solution was also increased to 75% (v/v) to reduce the stringency of RNA binding. The number and duration of washes post-hybridization and post-antibody incubation was substantially increased from several washes over two hours to several washes over more than six hours. The only notable effect of many of these alterations was the overall reduction of background staining visible in sense probe *in situ* hybridizations and to lengthen the amount of time required for antisense *in situ* hybridization background/ubiquitous staining to surface.

The staining with 1.2kb of labelled antisense and sense *At cnc* RNA probe is shown in fig. 6.8. *in situ* hybridization performed with antisense probe (fig.6.8A-C) has ubiquitous staining throughout the embryo. *in situ* hybridization performed with sense probe has staining in the yolk of the embryo, but the developing embryo is free of background staining (fig.6.8.D-F). In an attempt to control for non-specific binding, *in situ* hybridizations were performed both with sense probe and without any probe.

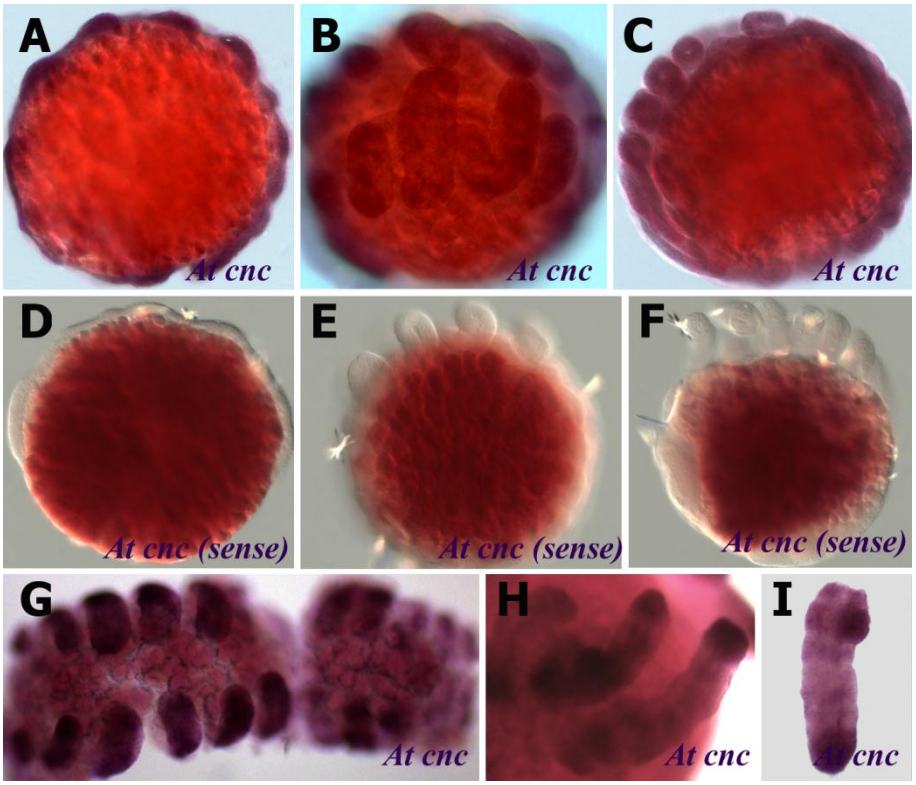

Fig.6.8. *in situ* hybridations with *At cnc* antisense and sense probes in *Achaearanea* embryos. Gene expression was detected by *in situ* hybridization. Anterior is to the left, ventral is top. (A-C) *in situ* hybridisation with *At cnc* anti-sense probe in (A) early germ band extending stage. (B) germ band retracting stage. (C) Late stage embryo. (D-F) *in situ* hybridisation with *At cnc* sense probe in (D) early germ band extending stage. (E) germ band retracting stage (F) Late stage embryo. (G-I) Alternative *in situ* hybridisation experiment that produced assymetric pseudo-expression patterns. (G) Ventral view. (H) close up of pedipalp and first leg appendage. (I) Dissected leg. Proximal is top.

Spider embryos that did not have any probe added produced no background staining at all (data not shown).

In embryos subjected to one week of hybridization and one week of antibody incubation, asymmetric spots of staining were detected. There was a relative increase in staining at the tips of appendages and the base of appendages (see fig.6.8G-I). A dissected leg appendage is shown in fig.6.8I. As the staining was asymmetric and non-reproducible between different embryos, this staining pattern was interpreted to be an artefact of that particular *in situ* protocol used.

6.3 Discussion

General overview of results

The homologue of *cnc* was discovered in *Achaearanea*. The short sequence obtained by degenerate PCR was extended by RACE. Alignment of the conserved regions of the protein sequence including the bZIP domain confirms the identity of the cloned gene as a CNC family member, most likely the orthologue of *cnc* present in single copies in other arthropod species. The identity of *Tc cnc* was confirmed by aligning the sequence of the bZIP domain with members of the CNC family and bZIP family of transcription factors. It is clear from the sequence comparison that the sequence obtained is nearly identical to other *cnc* orthologues in other arthropods and CNC family members in *C. elegans, H. sapiens* and is distinct from other bZIP family transcription factors such as *kay, Jra* and *CREB* in *D. melanogaster* and AP-1, FOS in *H. sapiens.*

No morphological structure specific expression pattern was detected by *in situ* hybridization in any of the numerous *in situ* hybridization experiments that were performed with labelled RNA probes of the antisense *At cnc* sequence. Positive control *in situ* hybridization experiments showed that the *in situ* hybridization protocol used was successful in detecting the transcripts of *At en, At Dll* and *At Dfd-1.*

Comparison of the staining revealed by the antisense *At cnc* probe compared to the sense probe reveals stronger relative ubiquitous staining. Staining with the sense probe was limited to the yolk cells at the interior of the embryo which is common for *in situ* hybridizations with all genes, sense or antisense, in this species. It is not possible to determine whether the ubiquitous staining observed with the antisense probe is signal or background. Ubiquitous expression may indicate that *At cnc* may be performing a housekeeping role that results in ubiquitous expression in all germ tissue. Lack of specific expression domains present in other arthropods, such as in the labrum, would be evidence of the loss of these expression domains. It is possible that the primitive function of *cnc* had no developmental role prior to acquisition of developmental roles in the mandibulate arthropod lineage. Although the function of a *cnc* homologue in *C. elegans*, *skn-1*, has roles in patterning mesodermal and endodermal structures during development, which suggests that developmental roles of *cnc* homologues may be ancestral (Bowerman et al., 1992; Walker et al., 2000).

The sense probe is not a quantitative control however, and the reasons for the non-specific background staining of *At cnc* antisense probes cannot be determined with any confidence based upon the *in situ* hybridization expression patterns alone.

Conserved housekeeping role of *cnc* in responses to environmental stresses

cnc has been shown to play important roles in a non-developmental context. Homologues of *cnc* have important roles in oxidative and xenobiotic stress responses that have shown to be conserved in diverse organisms.

The lack of an expression pattern in the *in situ* hybridization experiments described in this chapter (some representative embryos shown in fig.6.8) could represent the actual expression of *At cnc*. If this is the case, the developmental roles of *cnc* have been lost in *Achaearanea* and possible the chelicerate lineage. Alternatively, *cnc* had no developmental role prior to the acquisition of a developmental role in the mandibulate lineage. Much more work would have to be performed to show this is this case as it is very difficult to prove that a gene plays no role in development based upon a lack of gene expression.

CNC family members, Nrf2 in chordates and *cnc* in *Drosophila*, are activated in response to stress and are involved in oxidative stress responses in diverse organisms (Motohashi and Yamamoto, 2004; Sykiotis and Bohmann, 2008; Sykiotis and Bohmann, 2010). The Keap1/Nrf2 signalling pathway is activated in the fruit fly in response to oxidants and xenobiotics . Nrf2 acts in combination with Keap1 to promote organismal homeostasis, inducing anti-oxidant and detoxification responses. The Keap1/Nrf2 pathway is involved in aging. Antioxidant response elements (ARE) have been shown to be activated by Nrf transcription factors to regulate antioxidant stress responses. For example, in *Drosophila,* isoform C of *cnc* (CncC) has been shown to bind an ARE sequence upstream of the glutathione S-transferase gene (*gstD)* involved in the stress response (Sykiotis and Bohmann, 2008). Recently it has been shown that CncC in *Drosophila* has another role in regulating the 26S proteosome that is critically important for regulating the degradation of proteins and cell cycle control (Grimberg et al., 2011). CncC has also been shown to be necessary and sufficient for transcriptional responses to three xenobiotics: phenobarbital (PB), chlorpromazine, and caffeine (Misra et al., 2011).

Considering the involvement of CNC family members in these housekeeping roles in chordates such as mice and insects like *Drosophila,* it seems highly likely that *At cnc* will also perform such a role in *Achaearanea.*

In chapter four, it was observed that significantly increased mortality rates occurred in parental *Tc* cnc^{RNAi} experiments in *Tribolium*. As *cnc* is important for housekeeping roles in *Drosophila, Tc* cnc^{RNAi} may therefore affect organismal homeostasis, in particular the oxidative and xenobiotic stress responses, in adult beetles which could explain the significantly increased levels of mortality. It remains to be tested whether this is actually the case, but considering the conservation of the Keap1/Nrf2 signalling pathway between humans, mice and fruitflies, it is considered highly likely that *Tc cnc* is important for oxidative stress responses and proteolytic regulatory mechanisms.

What role does *cnc* perform in Chelicerates?

If the posterior collar domain of mandibulate cnc expression is missing from the first homologous walking leg segment of chelicerate groups, it would provide some evidence that the differentiation of the mandibular from the maxillary segment by *cnc* is specific to Mandibulata and is evidence that the mandible is a synapomorphy of that group. However, because there was no specific expression domain of *At cnc* observed, it is not possible to conclude that there is no L1 expression domain of *At cnc*, which would be indicative of a developmental role in patterning that segment.

It is reasonable to consider it unlikely that *cnc* will differentiate the first and second leg segments for two reasons. Firstly, the anterior boundary of *Dfd* homologues are expressed in the first leg segment and the anterior boundaries of the hox genes *Scr* and *ftz* are expressed in the second leg segment. Any L1 differentiating function of *At cnc* would probably be redundant. Secondly, the first and second leg appendages display little differentiation from one another.

This does not rule out the possibility of an appendage patterning role for *At cnc*. Indeed, the manner in which *At cnc* may function in the developing leg appendages, or how it interacts with Hox genes in these segments could provide clues as to how *cnc* evolved to pattern the mandible. For example in *Tribolium, Tc cnc* represses *Tc Dfd* and *mxp* in the mandibular segment and differentiates the mandible, which is protopodal in origin. Therefore, if *At cnc* is shown to interact with the Hox

genes in the limbs or if *cnc* is expressed in the protopodite of the limbs the path of evolution of *cnc* mandibular patterning function can be understood. *At cnc* could be involved in patterning the protopodite of the arthropod ancestor for example, and its function modified to pattern the mandibular protopodite.

There are non-mandibular *cnc* expression domains in mandibulates that are expressed in structures that are present in non-mandibulates, such as the labrum and the stomodeum. *cnc* performs a gap gene like role in the labrum of *Tribolium* and is responsible for patterning labral structures in *Drosophila.* The labrum is a conserved structure present in all arthropods. It was anticipated that *At cnc* might be expressed in the labrum. There was however no expression detected in the labrum.

A more distant outgroup to the mandibulates are the onychophorans. Degenerate PCR was performed to try to clone the *cnc* homologue present in an Onychophoran *Euperipatoides* to examine expression in this species. However, these experiments were not successful (data not shown). It would have been interesting to study *cnc* in the onychophoran, particularly regarding the conservation of the anterior expression domain of *cnc* in the labrum of insects and myriapods. The labrum has been hypothesized to be homologous to the primary antennae, or at least part of it, of Onychophorans (Budd and Telford, 2009; Eriksson et al., 2010). The labrum is appendage like in that it forms for two paired lobes that express leg patterning genes such as *wg* and the PD domain genes. The homeobox gene *six3* is expressed in the labrum and the primary antennae of onychophorans (Haas et al., 2001; Kimm and Prpic, 2006; Posnien et al., 2009). Expression of *cnc* in an onychophoran could also have been informative about the role of *cnc* homologues in the ancestor to arthropods.

It is something of a disappointment that I was unable to determine a specific expression pattern of the spider *cnc* homologue as it means that I was unable to demonstrate conclusively that *At cnc* had no developmental function in the L1 segment, and that *At cnc* has no expression pattern in the labrum. The labrum is a structure of uncertain origin and uncertain homology to other structures. The labrum is purported to be homologous to the antennae of onychophorans and of an appendage nature, it is of immense interest to arthropodologists (Budd and Telford, 2009; Posnien et al., 2009). *Tc cnc* was shown in chapter four to be necessary for the formation of the labrum, and it would have been a significant result if it was expressed

in the labrum of the spider indicating that it has a primitive function in patterning this interesting arthropod structure.

More research would need to be done both to determine if *At cnc* has lost all developmental functions, and whether it has retained a role in responses to xenobiotics and oxidative stress. An avenue of research that could be pursued is the detection of *At cnc* protein expression with a *cnc* specific antibody, which may be more sensitive to detect expression or may reveal specific protein expression domains.

It is entirely conceivable that *At cnc* has lost all developmental functions in the spider, but it is unfortunately difficult to test this hypothesis. One means of testing whether *At cnc* has lost developmental functions and is performing a housekeeping role, is to test relative expression levels throughout embryogenesis. Housekeeping genes are typically expressed at a constant level, in contrast to developmental genes which are up-regulated when activated to pattern embryonic structures. Detection of the relative abundance of transcripts by Quantitative PCR (QPCR) could show that *At cnc* is expressed at uniform levels at all stages of embryogenesis. Alternatively, QPCR could also determine at precisely which stage *At cnc* is upregulated during embryogenesis. This would facilitate the quest to obtain a tissue specific developmental expression pattern.

Chapter 7: Discussion

7.1 General overview of results

The primary aim of this research thesis was to study the development of a primitive mandible in a mandibulate arthropod. If the mandible is homologous between insects, crustaceans and myriapods (as hypothesized), it suggests that there may be mandible patterning genes that are shared between these groups. The mandible has probably evolved from a maxilla-like biramous limb present in crustaceamorphs like *Martinssonia* by modification of an endite into the characteristic mandibular gnathal edge. The *Tribolium* mandible retains the gnathal edge, with an incisor and molar process, which is the primitive characteristic that distinguishes the mandible from all other appendage types. It was therefore hoped that by studying the *Tribolium* mandible that three aspects of mandible evolution and development could be explored: i) Discover conserved mandible patterning genes. ii) See if the manner in which the mandible is patterned recapitulates mandible evolution from a maxilla-like precursor. iii) Homologize mandibular sub-structures (like endites and segments) to sub-structures on other appendage types.

The experiments performed in this study have provided some answers to the questions outlined above. The *Tribolium* homologue of *cnc* is required to pattern the mandibular segment and differentiates the mandible from maxillary identity. *Tc cnc* achieves this in part by repression of the Hox genes that are responsible for patterning the maxilla, *Tc Dfd* and *mxp. Tc Dfd* patterns protopodite structures in the maxilla and activates protopodite specific domains of expression, which may be similar to what is required to pattern the mandible (a protopodite). All of these results are similar to those observed in *Drosophila*. In *Tribolium* however*, Tc cnc* is activated by *Tc Dfd* in the mandibular segment whereas in *Drosophila, cnc* is activated independently of *Dfd*. Expression of *cnc* and *Dfd* are conserved in mandibulate arthropods. These observations indicate that the mandible patterning mechanism described for *Tribolium* is very likely to be conserved and probably originated once in the ancestor to all mandibulate arthropods. As the mandible has probably evolved from a maxilla-like

precursor, the acquisition of a maxilla-to-mandible differentiating role for *cnc* resembles the likely evolutionary history of the mandibular appendage.

I have provided evidence based on expression of the Notch signalling pathway, which marks the formation of arthropod appendage segments, that the *Tribolium* mandible consists of two segments, a subcoxa and a coxa. The incisor and molar process are derived from the outer and inner lobes respectively, which develop from a solitary endite located on the mandibular coxa. There are significant similarities of the subcoxa and the coxa of the mandible to the subcoxa and coxa of other appendages based upon the timing and location of PD domain genes expression relative to the expression of a member of the Notch signalling pathway. These similarities are evidence of serial homology between the subcoxa of the mandible to the subcoxa of the maxilla, labial and leg appendages.

After discussing the results of the previous chapters in more detail, I will discuss the implications of the serial homology of the mandible subcoxa and coxa to other appendage segments. I will then present a summary of the mandibular patterning mechanism of *Tribolium,* followed by an evaluation of the likely ancestral expression of the Hox genes in the ancestor to the mandibulate arthropods and a model of mandible evolution via the acquisition of a mandible patterning function of *cnc.*

Mandible patterning genes in *Tribolium*

It was shown in chapter four that *Tc cnc* differentiates the mandible from a maxilla probably in part by repressing the maxilla patterning Hox genes. The Hox genes that are required to pattern the maxilla, *Tc Dfd* and *mxp,* are repressed by *Tc cnc* in regions of the mandibular segment. This is similar to what is observed in *Drosophila*. As the mandible has probably evolved by modification of a maxilla-like appendage, genetic patterning of the mandible by *Tc cnc* recapitulates evolution of the mandible from a maxilla. The expression of *cnc* is conserved in the mandibular segment of diverse mandibulate arthropods, which suggests that *cnc* shares a similar function and that the mandible patterning function of *cnc* may have evolved once in the stem lineage of Mandibulata.

cnc is a promising candidate gene to have evolved mandible patterning function in the ancestor to all mandibulates. *cnc* is the only known gene that differentiates the mandible segment from other segments in *Tribolium* and *Drosophila*,

although I have shown in *Tribolium* that it is dependent on *Dfd* to achieve this function. The function of *cnc* has only been studied in two arthropods, *Tribolium* and *Drosophila,* and only one of them a typical mandibulate, *Tribolium*. Therefore, to show that *cnc* was responsible for differentiation of the mandibular segment from the maxillary segment in ancestral mandibulates, it has to be shown that this role of *cnc* is ancestral to Mandibulata and that it does not have any such role in the sistergroup of Mandibulata. A likely corollary of this is that the function of *cnc* will be conserved in diverse mandibulates. In species in which *cnc* is shown not to pattern the mandible, it would have to be demonstrated that this loss of function is a derived condition.

cnc represses *Dfd* in the both *Tribolium* and *Drosophila.* In chapter five I showed that in *Tribolium, Tc Dfd* is required to pattern the mandible (a protopodite) and the protopodite of the maxilla and that it activates protopodite specific gene expression, such as *Tc prd* and the proximal domains of *Tc dac* and *Tc Dll*. The mandible gnathal edge evolved from the endite on the protopodite of a limb and therefore the regulation of *Dfd* by *cnc* is particularly relevant regarding the hypothesized ancestral patterning function of *cnc* to differentiate the mandible gnathal edge from the maxillary endites.

In chapter five I showed that *Tc Dfd* patterns the endite and protopodite of the maxilla but does not control limb segmentation. *Tc Dfd* activates *Tc prd* and the proximal domain of *Tc dac* and *Tc Dll* but does not activate *Tc ser* expression. It was not possible to show if this is the case in the mandible, as the mandible is transformed to antennal identity and therefore shows antennal specific expression domains of these genes. However, it is reasonable to hypothesize that *Tc Dfd* performs a similar function in the mandibular segment and that *Tc cnc* modifies expression of these genes to mandible segment specific domains of expression. Alternatively, *Tc cnc* may activate these genes directly. More detailed genetic experiments are required to unpick these specific genetic interactions. If these specific genetic interactions were demonstrated in other mandibulates it would provide more proof that the mandible patterning mechanism is conserved. Below I discuss the mechanism of mandibular segment patterning and the likelihood of its conservation in other mandibulates by examining expression of the Hox genes that are required to patterned gnathal appendages in other arthropods.

The role of *cnc* homologues in non-mandibulates

In order to prove that *cnc* acquired a new role to pattern the mandible in the ancestor to all mandibulates, it is necessary to determine that this role is shared by other mandibulates. To show that the mandibular segment patterning role of *cnc* (homologous to the first leg segment of chelicerates) was acquired once in the lineage leading to the mandibulate arthropods, it is important to study the expression of *cnc* in non-mandibulates. As there is a reasonable chance that the function of *cnc* will not be the same in these outgroups (as they do not have a mandible), it is important to study as many outgroups as possible to have a better idea of the ancestral developmental role, if any, of *cnc.*

It has to be shown how *cnc* functioned in outgroups to the mandibulates. For example, it has to be shown that *cnc* does not in some way differentiate or pattern the segment homologous to the mandibular segment in non-mandibulates.

Unfortunately, I was unable to determine a specific expression pattern in the spider *Achaearanea* in order to show whether or not *cnc* has a first leg segment domain of expression in the spider. It is possible that *cnc* does not have a developmental role in the spider, either because embryonic roles of *cnc* function were never acquired in the chelicerate lineage or because developmental functions have been lost. To prove that a gene, like *cnc*, does not have a developmental role (as opposed to ubiquitous expression) by *in situ* hybridisation is difficult without additional evidence as there will be no specific expression pattern that would confirm that the *in situ* hybridisation experiment was successful.

One means to do this experimentally is to shown that levels of *cnc* transcription are maintained at constant levels throughout embryogenesis by quantitative PCR. If it is shown that *cnc* is not upregulated at any stage of embryogenesis, this would provide some support for the notion that *cnc* has no embryonic developmental role. Transcripts of *At cnc* were detected at all stages of embryogenesis by RT-PCR (with appropriate controls proving that there was no genomic DNA contamination), however, this is to be expected as *cnc* has a conserved role across metazoans in oxidative and xenobiotic stress responses. It is necessary to use a quantitative method of detecting transcript abundance to show that a gene does not show stage specific upregulation, and therefore a likely developmental role.

I was unsuccessful in my attempts to clone a homologue of *cnc* in an onychophoran, which is a more distantly related outgroup to the mandibulates.

The question is still open therefore as to whether *cnc* homologues in non-mandibulate arthropods have a developmental function, and it is not yet known whether *cnc* is expressed in the homologous segment to the mandibular segment, the first leg segment of chelicerates and onychophorans. Skn-1, the homology of *cnc* in *Caenorhabditis elegans,* has been shown to have embryonic developmental role in patterning mesodermal derived structures like the pharynx and body-wall muscle and intestines derived from endoderm (Bowerman et al., 1992; Walker et al., 2000)

Characterization of the mandibular endite.

In order to study the developing mandible in *Tribolium* embryos I studied the expression of genes i) that are expressed in the endites, ii) a gene that marks the developing segments of appendages and iii) the PD domain genes to identify appendage segments.

By studying genes that are expressed in the endites, *Tc prd* and *Tc dac,* I demonstrated in chapter two that the inner and outer lobes of the mandible correspond to the developing molar and incisor process respectively. Comparison of the expression of these genes in the mandible to the maxillary endites showed that these two lobes are very likely to be derived from one endite as the expression pattern in the mandible resembles the expression pattern seen in each endite of the maxilla.

Understanding that the gnathal edge is derived from one endite enables comparison of the gnathal edge, as a single endite, to other endites on other appendages. Machida hypothesized that the incisor and molar processes were homologous to the galea and lacinea respectively, which implies that the incisor and molar processes are derived from two separate endites. As the incisor and molar processes are derived from one endite as shown in chapter 2, Machida's hypothesis of mandibular endite homology is incorrect.

Locating the endite to a particular segment, creates the possibility of homologizing it to endites present on segments of other appendage types.

7.2 The mandibular subcoxa

I was interested in investigating any evidence of segmentation of the mandible by studying the Notch signalling pathway, in particular by studying the expression of *Tc ser* which is expressed in the distal part of each appendage segment. These results were shown in chapter three. Machida studied SEMs of the developing mandible of a bristletail and found evidence of a vestigial subcoxal/coxal segment boundary and suggested the subcoxa was homologous to the cardo of the maxilla (Machida, 2000). Study of the expression of *Tc ser* in *Tribolium* showed that the mandible is indeed divided into a subcoxa and coxa segment. These segments do not form a joint in the larval or adult mandible. The presence of a ring of *Tc ser* expression in the mandible provides molecular evidence of a subcoxal/coxal division in the embryonic mandible. This result supports Machida's hypothesis that the mandible is subdivided into two segments. The mandible gnathal edge derived from the endite – both incisor and molar processes - is therefore present on the more distal coxal segment of the mandible.

Expression of *Tc ser* suggests that there is a subcoxal segment of the *Tribolium* mandible. This subcoxa has fused with the coxa to form the mandible present in *Tribolium*. Other apparently unsegmented mandibles may, like *Tribolium,* also have hidden subcoxal segments in their embryonic stages. Only some diplopods (millipedes) possess mandibles with an obvious subcoxal segment in postembryonic stages. Apart from the diplopods, there is no visible subcoxa present on the mandibles of any other mandibulate arthropod. It remains to be tested whether there is evidence of a hidden subcoxal segment present in other mandibles (shown in fig. 7.1G,D and fig.7.2C) by studying the Notch signalling pathway. Interestingly, if the presence of a mandibular subcoxa is primitive the segmented diplopod mandible could represent the ancestral state although this would require multiple independent losses of the subcoxa in other mandibulates.

Homology of the subcoxa and coxa of the mandible and maxilla

The presence of a mandibular subcoxa means that it is possible to homologize it to the cardo of the maxilla. This is assuming that both subcoxal segments are primitive characters that were present in the ancestral gnathal appendage (see fig. 7.1A). The expression of *Tc ser* was compared to the expression of the PD domain

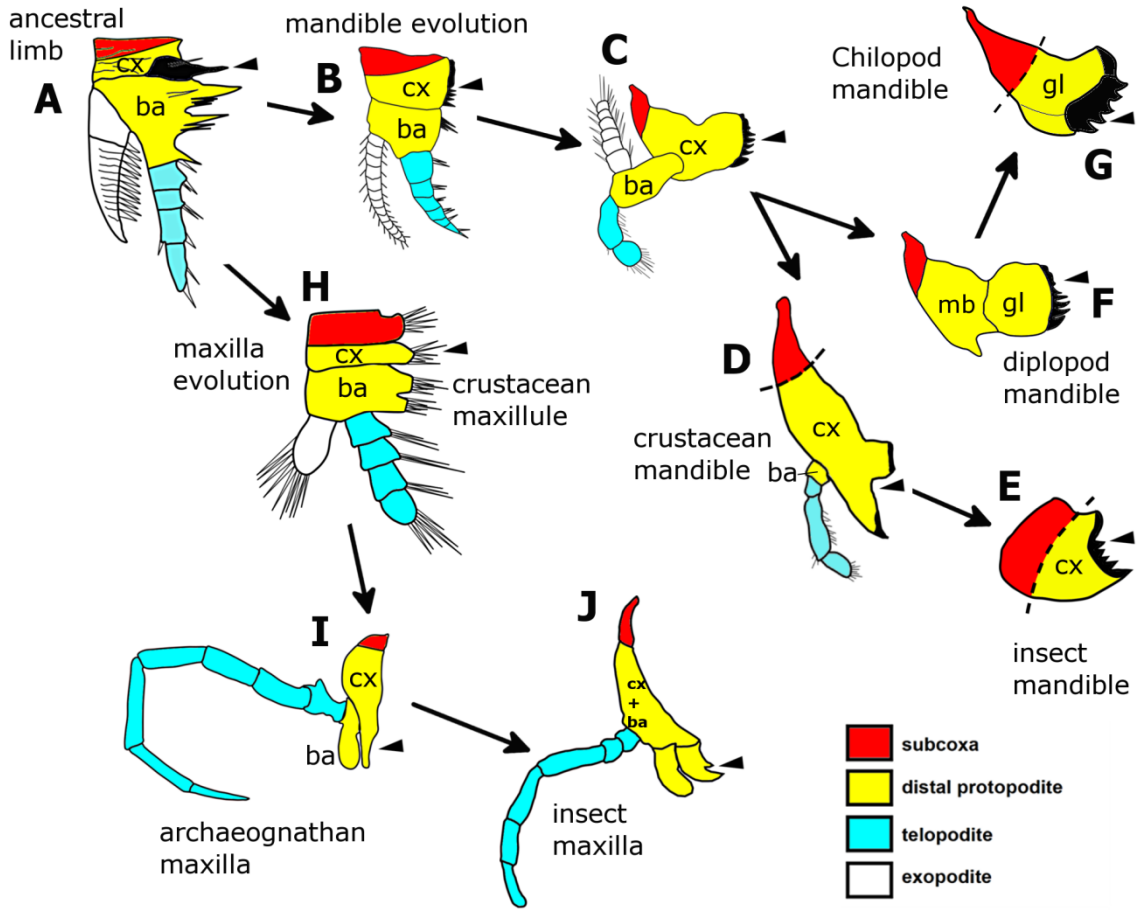

Fig.7.1 Evolutionary implications of the serial homology of the cardo to the subcoxa of the mandible. Schematic of evolution of the mandible and maxilla from an identical serially homologous limb. Two evolutionary paths from a common serially homologous limb are illustrated, the mandible and maxillary evolutionary paths. The coxa (cx) and basis (ba) are indicated. The telopodite is shown in blue and the protopodite is shown in yellow. Dotted line indicates embryological subcoxa/coxa division that does not result in a segment boundary. If the subcoxa (red segment) is homologous between the mandible and maxilla, it implies that the ancestral protopodite consisted of three segments. Serially homologous endites present on the coxa are indicated with an arrowhead. (A) Hypothesized ancestral biramous appendage from which the mandible and maxilla evolve. Protopodite has subcoxa, coxa and basis. (B) Hypothesized ancestral mandible, based on Cambrian crustacean *Bredocaris* mandibular limb morphology. (C) Hypothesized ancestral mandible. (D) Crustacean mandible with telopodite palp present and hypothetical division into subcoxa and coxa. (E) Unsegmented Insect mandible with hypothetical subcoxal/coxal division. (F) Segmented diplopod mandible with separate gnathal lobe (gl) attached to the mandible base (mb) (G) Chilopod mandible with a hypothetical subcoxal division and gnathal lobe fused with the mandible base. (H) Crustacean maxillule (homologous to maxilla. (I) Archaeognathan hexapod maxilla with three segmented protopodite. (J) Insect maxilla with a two segmented maxilla with the coxa and basis fused to form one segment, the stipes.

genes in order to identify each appendage segment. There are significant similarities of PD domain gene expression that are suggestive of serially homologous relationships between the subcoxa of the mandible, maxilla and labial appendages.

By comparing endites together with their segmental affinities it is possible to demonstrate homologous relationships. The insect maxilla, consisting of two segments (cardo and stipes), has probably evolved from a three segmented archaeognathan maxilla (subcoxa, coxa and basis) (compare fig. 7.1I and 7.1J)

The molar and incisor processes derived from an endite present on the mandibular coxa (see fig. 7.1E and 7.2D). If the maxillary coxa of archaeognathans (see fig.7.1I) is homologous to the mandibular coxa, it suggests that the mandibular endite is homologous to the lacinea endite which is present on the coxa of the maxilla.

Serial homology of the subcoxa and coxa of the mandible and legs

Boxshall has commented that if the pleuron of the leg has a subcoxal origin then it is possible to serially homologize the leg protopodite segments to other appendages (Boxshall, 2004). The expression of *Tc ser* indicates that there is a subcoxa segment in the leg that develops into the pleuron in the larva confirming the subcoxal origin of the pleuron. Therefore there is a possibility that this putative subcoxa segment can be serially homologized to protopodite segments of the gnathal appendages. An evolutionary scenario which illustrates the serial homology of the subcoxa from a primitive three segmented protopodite to the insect mandible, maxilla and leg is illustrated in fig.7.2.

There are numerous similarities between the developing subcoxa and coxa of the *Tribolium* leg to the subcoxa and coxa of the gnathal appendages that suggest serial homology. The coxal-2 domain of *Tc ser* expression is activated at the same time in the maxilla, labial and leg limb buds. The proximal domain of *Tc dac* is activated with the coxal-2 domain of *Tc ser.* The subcoxal-1 domain of *Tc ser* is activated at the same time in all post-antennal appendages. There are also similarities of the maxilla telopodite to the developing leg. For example, in the leg and maxilla, *Tc Dll* and *Tc hth* are co-expressed with the trochanteral-3 *Tc ser* domain of *Tc hth.*

Accepting all these similarities of gene expression as evidence of serial homology, the leg coxa would be serially homologous to the maxillary stipes, the mandibular coxa and to the labial prementum. The leg subcoxa would be serially homologous to the maxillary cardo, the mandibular subcoxa and the labial postmentum.

However, there are some subtle differences between the proximal domains of *Tc dac* of the leg appendages compared to the gnathal appendages. These differences may reflect fundamentally different patterning mechanisms of these coxal segments, or may result from the different morphology of the limbs based on the presence of endites in the gnathal appendages. There are in fact three domains of expression of *Tc*

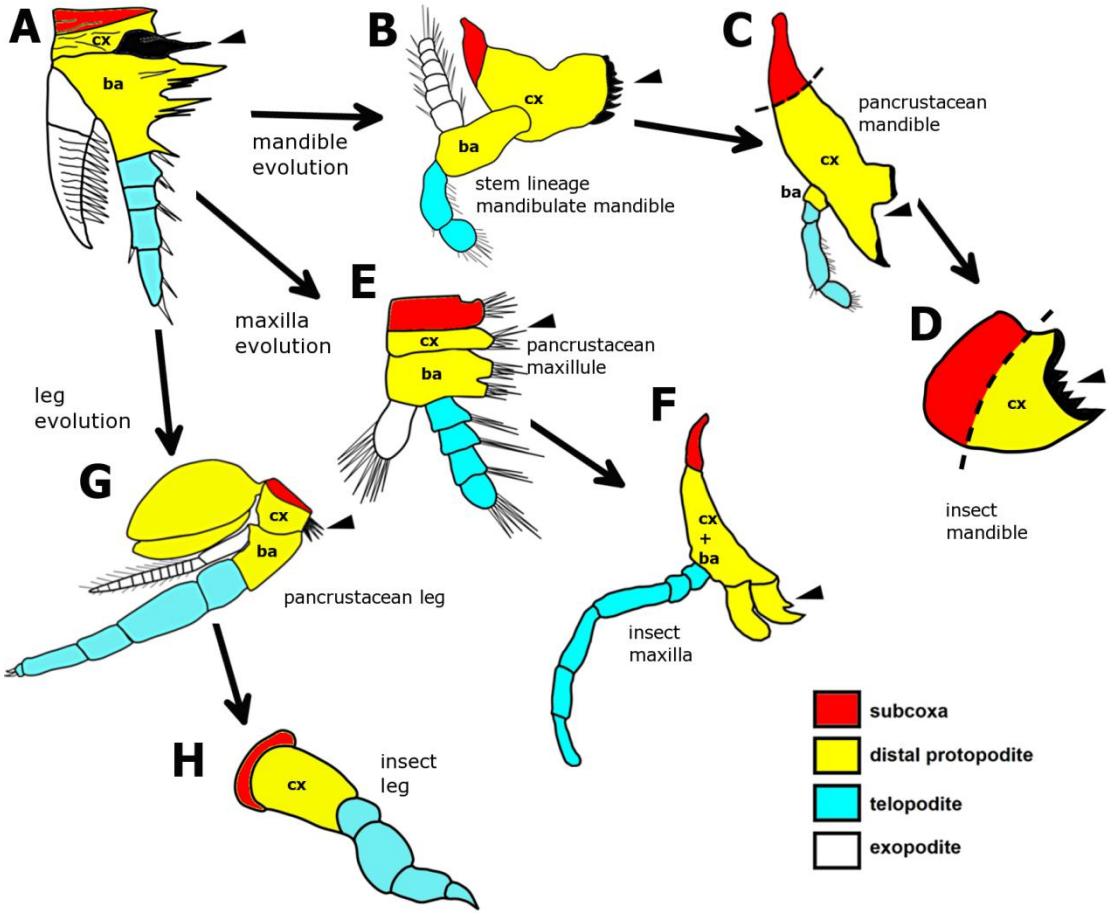

Fig.7.2 Serial homology of the mandibulary subcoxa to the maxillary cardo (subcoxa) and leg pleuron (subcoxa) of insects. Serial homology of the subcoxa of the insect mandible (D), maxilla (F) and leg (H) requires that the subcoxa is derived from the same proximal segment on the ancestral biramous limb (A). In this scheme, the insect appendages (D,F,H) evolved through primitive precursor appendages present on a hypothetical pancrustacean ancestor (C,E,G). This scheme homologises the mandibular endite to a single endite present on the coxa segment of the maxilla (arrowhead). Three evolutionary paths of three beetle appendages (mandible, maxilla and leg) from an ancestral biramous limb (A). (A) Hypothetical primitive post-antennulary limb with a three segmented protopodite on a stem lineage mandibulate based on limb of *Martinssonia elongata.* (B-D) mandible evolution. (B) Hypothetical stem lineage mandibulate arthropod mandible with two palps and mandible with hypothetical subcoxal/coxal division. (C) hypothetical pancrustacean mandible mandible with hypothetical subcoxal/coxal division and a palp. (D) Insect dicondylic mandible with hypothetical subcoxal/coxal division. (E-F) maxilla evolution. (E) hypothetical primitive pancrustacean maxillule. (F) insect maxilla with fused coxa and basis segments. (G-H) leg evolution. (G) hypothetical pancrustacean primitive biramous limb. (H) Insect leg with subcoxa derived pleuron. Dotted line indicates hypothetical embryological subcoxa/coxa division that does not result in a segment boundary in larval or adult forms. If the subcoxa (red segment) is homologous between the mandible and maxilla it implies that the ancestral protopodite consisted of three segments: subcoxa, coxa and basis. If the subcoxa of the insect leg (H) is homologous to the subcoxa of the insect maxilla (F) and mandible (D) it requires that these segments were present in the evolutionary lineages leading to all this limbs from an ancestral limb with a subcoxa present (A).

dac in the developing leg (Prpic et al., 2001)*, a* distal domain in the femur, a larger proximal domain which overlaps with the coxal-2 domain of *Tc ser* and another smaller spot of expression which is co-expressed with the subcoxal-1 domain of *Tc ser* (data not shown)*.* The proximal domain of *Tc dac* expression in the leg is co-expressed with the coxal-2 domain of *ser* expression throughout development. This is in contrast to *Tc dac* expression in the gnathal appendages where the proximal expression domain is expressed in between the coxal-2 and subcoxal-1 domains of *Tc ser* expression

domains in the developing coxa. This difference could be because of the presence of endites in the coxa of the gnathal appendages which are lacking in the leg coxa.

Serial homology of the subcoxa of the mandible to the subcoxa of the maxilla

Arthropod post-antennal appendages evolved from serially homologous biramous limbs. At some point in the evolution of the mandible and maxilla, the limbs were identical in structure and then diverged to evolve into the myriad forms of mandibles and maxillae that are present today (see fig.7.1).

One reasonable account of mandible and maxilla evolution posits that the ancestral protopodite on these appendages consisted of a two segmented protopodite, the coxa and basis. There were likely to be multiple endites present as this is commonly found in stem lineage crustaceans like *Martinssonia* and Phosphatocopida and stem lineage branchiopods like *Rehbachiella* (Muller and Waloszek, 1986; Waloszek, 1993; Siveter et al., 2001). The mandibles of myriapods, insects and some (especially terrestrial) crustaceans have lost both palps. The gnathal edge of the mandible is considered to be present on the proximal protopodite segment, which is the coxa (see fig 1.5), as the majority of mandibles do not have a subcoxal segment.

The serial homology of the subcoxa of the mandible and maxilla would suggest that the primitive mandible and maxilla originally had three protopodite segments, the subcoxa, coxa, and basis (fig. 7.1A). Maxillary protopodites with three segments are present in crustaceans (fig. 7.1H) and archaeognathan hexapods (fig.7.1I).

Evolution of the insect mandible from an ancestral maxilla-like precursor with a three segmented protopodite (see fig. 7.1A) could occur as follows (see fig.1.A-G): The proximal endite is modified to form the mandible gnathal edge (fig. 7.1B). The basis is reduced in size (fig.7.1C). The exopodite palp is lost, the basis is further reduced in size and the subcoxa fuses with the coxa (fig.7.1D). Finally, the telopodite palp is lost (fig.7.1E).

The subcoxa and coxa fuse such that external evidence of segmentation is lost and form the coxa which is evident in mandibles of the majority of living mandibulates. Study of the embryological development of the *Tribolium* mandible, as shown in chapter three, however reveals evidence of segmentation in the form of a subcoxa-coxa boundary and a subcoxal *Tc ser* domain of expression.

Hypothesized evolution of a maxilla protopodite with three segments is shown in fig. 7.1. Evolution from a three segmented maxilla protopodite does not require addition of segments in different lineages, but rather would be typified by loss and fusion of segments, for example, the insect maxilla has evolved by the fusion of the coxa and basis to form the stipes (fig. 7.1J).

If the subcoxa of the mandible and maxilla of *Tribolium* are not serially homologous, the similarity of *Tc ser* expression in the protopodite requires an explanation. It is difficult to speculate without knowing the precise functions of the proximal domains of PD domain genes but the similarity of the first two ring expression domains of *Tc ser* could reflect similarities of the gene regulatory network of limb patterning that has nothing to do with a serial homologous relationship. The gene regulatory network could have evolved in the lineage leading to *Tribolium* such that the similarities observed between appendages merely reflect the similarity of the patterning mechanism for limbs in general.

Criticism of serial homology hypotheses

A trend in evolutionary development is to make inferences of serial homology between appendages based upon data obtained from one species. The reasons for this are understandable, as it is often difficult enough to study one organism in a laboratory let alone the several required to make strongly supported inferences about ancestral character states. Machida's hypothesis of serial homology between the mandible and maxilla is based upon evidence from one species of bristletail (Machida, 2000). Prpic's hypothesis of the serial homology of the complete mandible to the coxa was based solely upon the expression of the proximal domain of *Tc dac* in *Tribolium* (Prpic et al., 2001). The present study is also susceptible to this criticism, by comparing the expression of *Tc ser* and the PD domain genes in different appendages.

It is easy to overinterpret apparent similarities of expression from one organism without providing the proper phylogenetic context of the evolution of these limbs. However, the combination of such diversity of appendage forms, the 100 million year ghost lineage of myriapod fossils and the difficulty of interpreting Cambrian arthropod appendage segments makes it difficult to infer the likely primitive character state of the ancestral mandible.

Certain key taxonomic relationships are still lacking, which would help in understanding the polarity of character states, that is to say, whether they are

primitive or derived. There is little consensus about the sister group to the hexapods within Pancrustacea (Edgecombe, 2010; Jenner, 2010).

Therefore creating an evolutionary scheme (like fig. 7.1 and 7.2) by placing appendages of stem group mandibulates through crown group mandibulates in a linear series of limb evolution is speculative. These hypotheses of serial homology are conditional on very particular arrangements of taxa in a linear series and are not robust to relatively minor revisions of arthropod phylogeny, or reinterpretations of particular fossils.

Confirmation of suggested homologous relationships requires evidence from more taxa, and for these characters to be mapped onto a phylogeny to determine homology or convergence. The study of PD domain genes together with the notch signalling pathway to mark developing segments complements morphological and palaeontological studies and could provide more objective criteria to establish homology.

7.3 Molecular development of the mandible

Conserved mandibular segment patterning genes in *Drosophila* and *Tribolium*

Results from chapter four have shown that the mandibular differentiating function of *cnc* by repressing *Dfd* is conserved in *Tribolium*. Despite the obvious larval and adult morphological differences, there are numerous conserved genetic functions and interactions between *Tribolium* and *Drosophila* in the gnathocephalon of the developing embryo. This indicates that the function of genes that pattern the gnathocephalon of *Tribolium* and *Drosophila* are conserved, and is evidence that mandibular patterning mechanisms may be conserved in other mandibulates.

The similarities are evident in the expression of genes, shown in fig.7.3A-C**.** The Hox genes *Dfd* and *pb* are expressed in similar domains (fig. 7.3B), as are *cnc* and *Dfd,* with repression of *Dfd* from the mandibular appendage (fig. 7.3A). There is similarity in the expression of *prd* and *Dll* in the proximal and distal regions of the maxillary lobe. *Tc cnc* also represses *Tc Dll* and the Hox genes *Dfd* and *mxp* (fig. 7.3C).

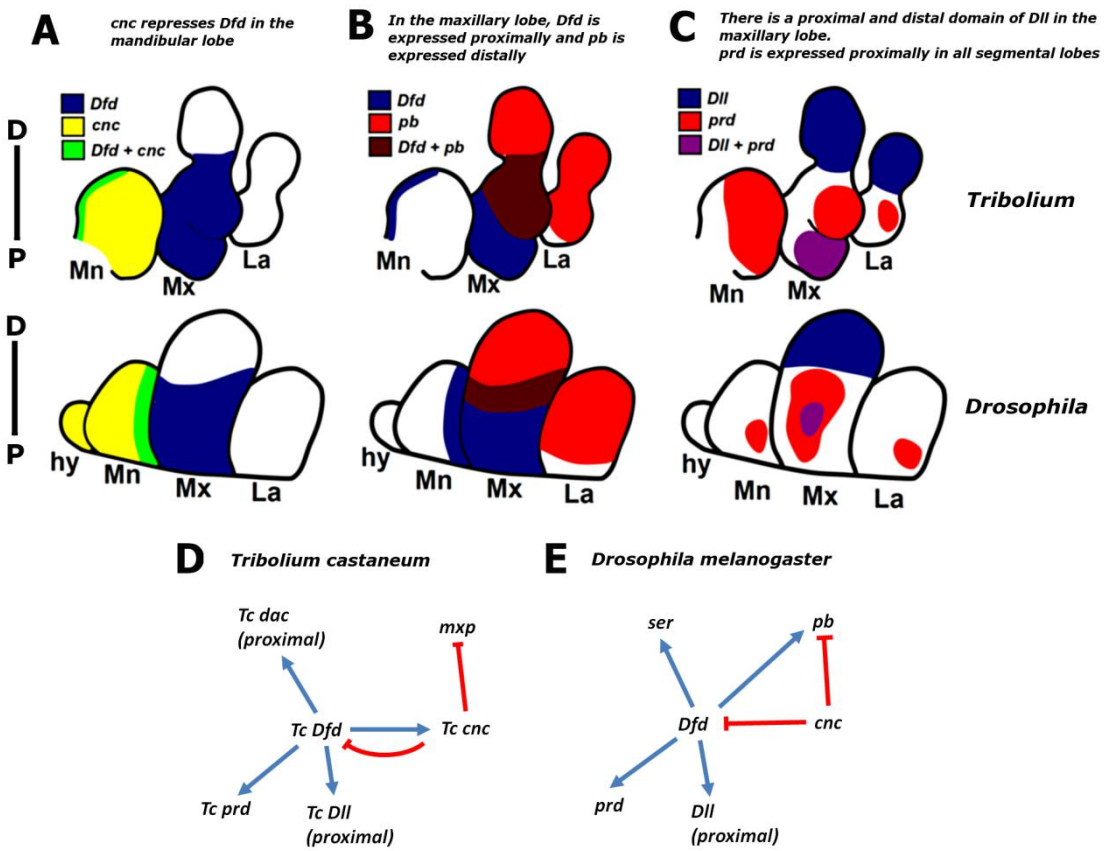

Fig.7.3 Comparison of gene expression and gene regulation in the gnathocephalon, the mandibular, maxillary and labial segments of *Tribolium* and *Drosophila*. (A-C) Conserved expression of genes in the mandibular, maxillary and labial segments of *Tribolium* and *Drosophila. Tribolium* gnathal lobes are on top, *Drosophila* gnathal lobes are on the bottom. Expression patterns are very similar between both species, with similarities in both segmental expression domains and proximal-distal domains. Proximal (P) and distal (D) axis are indicated. (A) Expression of *cnc* and *Dfd.* (B) Expression of *Dfd* and *pb.* (C) Expression of *Dll* and *prd.* (D) Summarized genetic interactions of *Tc Dfd* and *Tc cnc* in *Tribolium.* Interactions are similar to *Drosophila,* although *Tc dac* upregulates the proximal domain of *Tc dac* in the maxillary segment in *Tribolium* which does not occur in *Drosophila.* (E) Summarized genetic interactions of *Dfd* and *cnc* in *Drosophila.* Interactions are similar to *Tribolium. Dfd* activates *ser* and *pb* which does not occur in *Tribolium.*

There are several shared genetic interactions between *Tribolium* and *Drosophila* that are representative of their expression patterns (fig. 7.3D,E). In both species, *Dfd* patterns proximal structures that are derived from the gnathal lobes and regulates genes that have expression domains on the proximal part of the gnathal lobes. The proximal domain of *Dll* and the maxillary *prd* domain are regulated by *Dfd.*

There are some differences, like the regulation of *ser* by *Dfd, and mxp* by *cnc* which occur in *Drosophila* but not in *Tribolium.* More striking is the activation of *cnc* by *Dfd* which I have shown occurs in *Tribolium* but not *Drosophila.*

The phylogenetic relationship between these two members of the Holometabola has been examined recently in a phylogenomic study that showed that hymenopterans are more distantly related to Diptera than are the Coleoptera (Savard

et al., 2006). This indicates that the genetic mechanisms patterning the mandibular and maxillary segments are common to the Coleoptera, Diptera and Lepidoptera but cannot necessarily be extrapolated to the more distantly related Hymenoptera. Considering the conserved expression of *cnc, Dfd* and *pb* in diverse mandibulates, the mechanisms of patterning the mandibular and maxillary segments may also be conserved across mandibulates.

How *Tc cnc* and *Tc Dfd* pattern the mandible in *Tribolium*.

In this section, based upon evidence from chapters four and five, an outline of mandibular segment patterning in *Tribolium* will be presented. There is some consideration given to the role that *cnc* plays in patterning the mandibular segment in *Drosophila* as the experiments performed on the molecular developmental functions of *cnc* have revealed particular details that may be of significance for all taxa that have mandibular segments patterned by *cnc.* This hypothetical outline of mandible patterning may resemble the primitive functions of these genes in stem lineage mandibulates, with some differences concerning the presence of primitive mandibular palps. Following this section I discuss the likelihood of the conservation of this mechanism by comparing Hox gene expression in diverse mandibulates and non-mandibulates.

Here is a hypothetical scheme of the mandible patterning functions of *Tc cnc* and *Tc Dfd* (see fig. 7.4)*:*

1) *Tc Dfd*, as one of its roles as a Hox gene, is required in the mandibular segment to repress antennal development and establish the post-antennal appendage P/D axis, as defined by the PD domain genes (fig. 7.4A).

2) *Tc Dfd* activates *Tc cnc* in the mandibular segment. *Tc cnc* begins to differentiate the mandible from the maxilla. *Tc cnc* represses palp development in the mandibular segment by repressing the palp domain of *mxp(pb)* (fig. 7.4B).

3) *Tc Dfd* patterns the protopodite, including the endites of the mandible and maxillary segments (fig. 7.4C).

4) As soon as the endites start to develop, *Tc cnc* represses *Tc Dfd* from the mandibular endites, and *Tc cnc* differentiates the mandible endite from the maxillary endites (fig. 7.4D).

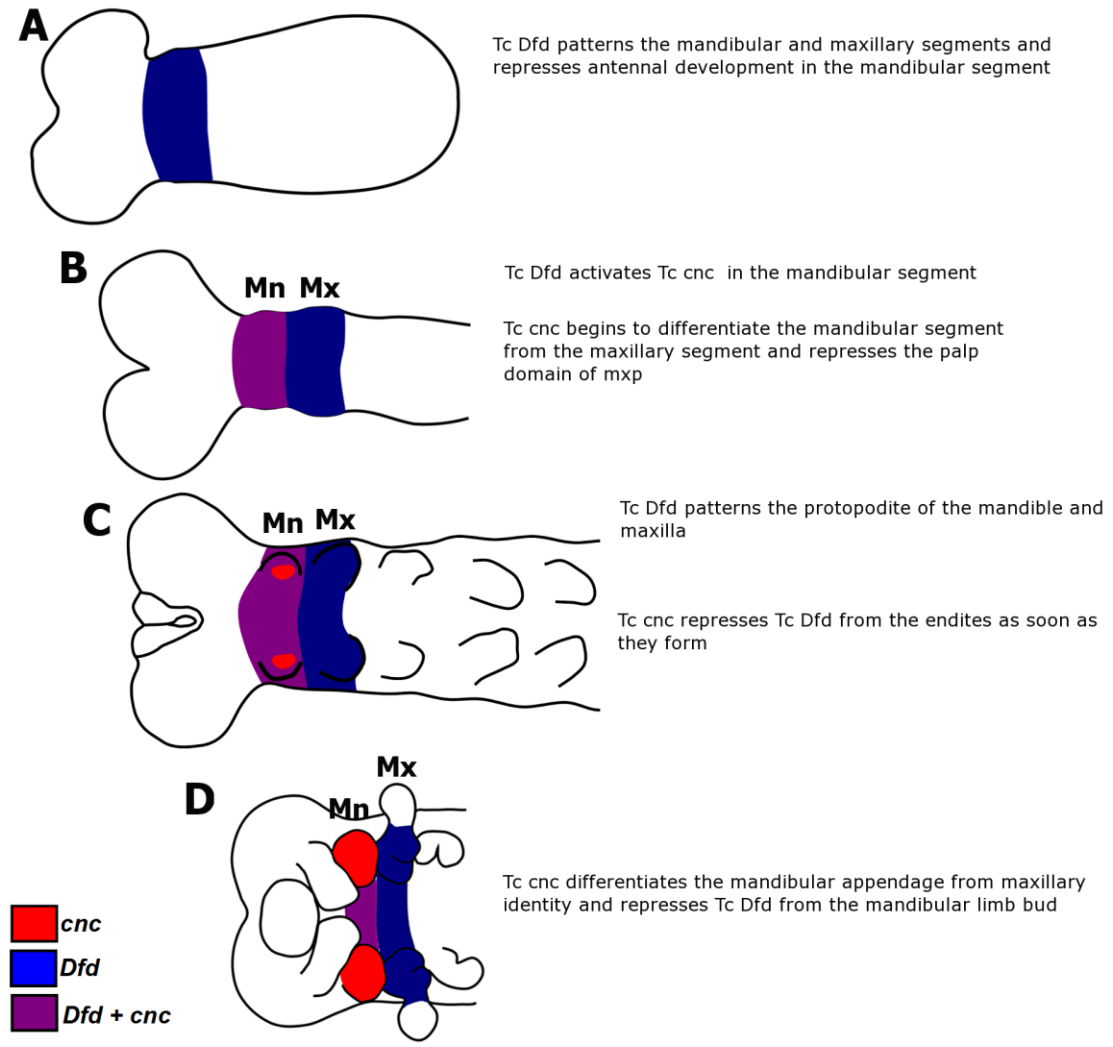

Fig.7.4 A model of *Tc cnc* and *Tc Dfd* mandibular and maxillary patterning functions in *Tribolium castaneum*. (A) *Tc Dfd* represses antennal development and establish the post-antennal appendage P/D axis, as defined by the PD domain genes. (B) *Tc cnc* is activated by *Tc Dfd* in the mandibular segment. Tc cnc begins to differentiate the mandible from the maxilla, and represses the ectodermal telopodite domain *mxp*. (C) *Tc Dfd* patterns the protopodite, including the endites of the mandible and maxillary segments. As soon as the endites start to develop, *Tc cnc* represses *Tc Dfd* from the mandibular endites, and Tc cnc differentiates the mandible endite from the maxillary endites. (D) *Tc Dfd* expression is repressed from the majority of the mandibular limb bud.

Hox genes have been shown to repress antennal identity (Brown et al., 2000). It can be inferred that the activation of the post-antennal appendage P/D axis pathway is achieved by Hox genes. In the maxillary segment this role is performed by the presence of *Tc Dfd* and *mxp*. In the mandible, *Tc Dfd* would perform this function.

It was shown in chapter four that *Tc cnc* is activated in the mandibular segment by *Tc Dfd*. It was also shown that *Tc cnc* represses *mxp* expression in the mandibular segment and represses telopodite development which indicates that at an early stage *Tc cnc* is functioning to differentiate the mandibular segment from the maxillary segment, as *mxp* is expressed in limbs at an early stage of development.

Tc Dfd promotes protopodite development and patterns the endites in the maxilla, probably by up-regulating or activating gene expression such as the proximal domain of *Tc dac* and *Tc prd*, and the proximal domain of *Tc Dll.* These results were described in chapter five. The proximal domain of *Tc dac* is expressed early in the mandibular and maxillary limb buds, as soon as they start to form. It is assumed, that *Tc Dfd* functions in a similar manner in the mandible however this has to be tested.

As soon as the endites of the mandible and maxilla start to develop, *Tc cnc* represses *Tc Dfd* in the mandibular endite. This is evident in comparison of *Tc cnc* and *Tc Dfd* expression, as *Tc Dfd* expression is repressed from the position of the endite during early limb stages of embryogenesis shown in chapter four.

Tc cnc at this stage differentiates the mandibular endite from the maxillary endite. *Tc cnc* promotes an alternative developmental pathway of the mandibular endite, which is evident in the differential expression of *Tc prd* in the mandible compared to the maxilla. Knock down of *Tc cnc* resulted in a reiteration of the maxilla domains of *Tc prd* expression.

One important piece of evidence lacking from the framework above is evidence that shows *Tc cnc* is an activator of mandibular patterning genes in *Tribolium*, and whether *Tc cnc* is sufficient to pattern mandibular structures. *cnc* may play such a role in mandibulate arthropods like *Tribolium*. *Tc cnc* clearly functions to differentiate a mandible from a maxillary appendage, and it has been shown to have activator functions in *Drosophila*. The mandibular appendage obviously requires genes to pattern it. It is highly unlikely that repression of *Tc Dfd* by itself will result in the patterning of a mandibular appendage. Other genes will be activated to pattern the appendage. If *Tc cnc* is not directly involved in the activation of these mandibular patterning genes, then somehow *Tc cnc* indirectly activates them as *Tc cnc* is required to differentiate the mandible from a maxilla.

Numerous lines of evidence suggest that *cnc* in *Drosophila* functions as an activator and that any repression of Hox genes by *cnc* is likely to be indirect (Veraksa et al., 2000). Cnc possesses a strong activating domain. The *cnc* binding sequence, for example, when placed next to *Dfd* response element results in increased Dfd activity, not a reduction of activity which would be expected from a repressor.

cnc has been shown to repress both *Dfd* transcription and *Dfd* activity even when *Dfd* transcripts are present. It is hypothesized that Cnc represses Dfd protein

which prevents the activation of the *Dfd* autoactivation circuit. This would result in repression of transcription. Cnc has been shown to physically interact with Dfd protein, although the significance of this is not known.

It has been noted that *cnc* has a function in patterning some mandibular segment derived structures independently of *Dfd* (Veraksa et al., 2000). Ectopic activation of *cnc* in *Drosophila* embryos results in the production of hypopharyngeal structures (the T-ribs), which are derived from the hypopharyngeal lobes, which are part of the mandibular segment (Economou and Telford, 2009). These ectopic mandibular segment derived structures are present along the ventral midline of the *Drosophila* embryo where there is no *Dfd* expression. This result indicates that *cnc* is necessary and sufficient to pattern some mandibular segment derived structures, independently of *Dfd.*

An instructive experiment would be to test whether *Tc cnc* is sufficient to pattern mandibular structures by ectopically activating *Tc cnc* throughout the developing *Tribolium* embryo. If ectopic mandibular structures were present ubiquitously, it would prove that *Tc cnc* was a master controller gene sufficient for at least some aspects of mandible development. If ectopic mandibular structures were formed in the maxilla and labial endites only, it would show that endite formation is required prior to *Tc cnc* mandible patterning function. If mandibular structures were only present in the maxillary segment then it would show that *Dfd* is required for *cnc* to pattern mandibular segment structures. This experiment would answer the question of whether *Tc cnc* is directly responsible for activating the alternative endite developmental pathway of the mandibular endite.

Another experiment that could show similarities between *cnc* function in *Drosophila* and *Tribolium* would be to ectopically express *Tc cnc* in *Drosophila* embryos to see if it could rescue the wild type phenotype in *cnc* mutants and also to see if ubiquitous ectopic activation resulted in the production of ectopic hypopharyngeal lobe derived structures.

The more that is understood about the molecular mechanism of mandibular patterning, the more that is likely to be understood about the evolution of the mandible by comparison to known genetic functions. Comparison to other taxa would also be easier and more informative as a result.

7.4 The role of *cnc* in the ancestor to all mandibulates

Expression of *cnc* in diverse mandibulate arthropods such as the myriapod *Glomeris* and other insect species suggests that the mandible patterning function of the posterior domain of *cnc* function is conserved in mandibulates.

In *Tribolium* it has been shown that *Tc Dfd* patterns the mandible and the protopodite of the maxilla (Brown et al., 2000). *mxp* (the *pb* orthologue) patterns the palps of both the maxilla and labial appendages (DeCamillis et al., 2001). *Cx* (the orthologue of *Scr*) patterns the protopodite of the labial appendage and the first thoracic segment (Curtis et al., 2001).

The expression patterns of *Dfd, pb* and *Scr* in particular segments and parts of appendages are conserved in numerous arthropod species. This suggests that the patterning of the mandible and maxilla in different taxa is conserved. But there are also notable differences.

Some of these differences clearly relate to the derived structure of some gnathal appendages, for example, numerous maxillary appendages have lost the palp and therefore have lost *pb* expression. Other differences are harder to account for and may represent derived states. For example, there is diversity of *Scr* expression patterns across mandibulates.

From a consideration of the expression of these genes, the hypothetical expression of these genes in a non-mandibulate and a mandibulate ancestor to all mandibulate arthropods and will be described (shown in fig. 7.6B,C).

Conserved expression of *Dfd* in maxillary protopodites

Dfd expression is conserved in numerous insect, crustacean and myriapod species. *Dfd* expression is located in the mandibular and maxillary segments. Typically there is a retraction of *Dfd* expression during development in the mandibular endite that suggests *cnc* represses *Dfd* in the mandibular appendage as described in chapter four (Rogers et al., 2002) (Kokubo et al., 1997; Abzhanov and Kaufman, 1999a; Walldorf et al., 2000; Hughes and Kaufman, 2002b; Rogers et al., 2002; Mito et al., 2008).

In the majority of these species, *Dfd* is expressed in the mandibular and maxillary segments. Expression is strongest in the maxillary protopodite and repressed from the mandibular limb bud, although there are exceptions. In the crustacean

Porcellio, Dfd expression is limited to the mandibular segment. The hemipteran *Oncopeltus,* that possesses derived styllate mouthparts and has lost the mandibular endite, has ubiquitous expression of *Dfd* throughout the appendage (Hughes and Kaufman, 2000; Rogers et al., 2002).

In *Lithobius,* the homologous segment to the labial segment is the second maxilla. The appendages on this segment are fused to the sternites to form a single coxosternite. Interestingly, *Dfd* is expressed in the protopodite of the second maxilla appendage as well as the mandible and the first maxilla.

A recent study investigating *Dfd* expression in an onychophoran *Euperipatoides* shows that *Dfd* is expressed in the proximal region of each walking limb bud (Eriksson et al., 2010). This result suggests that *Dfd* expression in the base of the mandibular and maxillary limbs is ancestral (see fig. 7.5A).

However, functional studies on other mandibulates are required to prove the hypothesis that the protopodite patterning function of *Dfd* represents the ancestral state. Crustacean taxa are particularly poorly sampled, especially considering the diversity of gnathal appendages present.

Conserved expression of pb in maxillary telopodites

pb expression is conserved in maxillary telopodites which suggests that it is required for patterning these structures (Abzhanov and Kaufman, 1999a; Hughes and Kaufman, 2002b; Rogers et al., 2002; Janssen and Damen, 2006).

Although *pb* orthologs are commonly expressed in the telopodites of maxillary appendages, there are exceptions, which may relate in part to the morphology of the maxillary appendages found in those species. A number of studied organisms do not have maxillary palps and consequently do not have *pb* expression in the maxilla. In the case of *Glomeris*, the maxillary appendages have evolved into a structure called the gnathochilarium which consists of maxillary appendages which have lost their palps and fuse ventrally. A similar situation exists in the Isopod *Porcellio*, the maxilla has lost its palp, or it has become highly reduced. *pb* has a small domain of expression in the second antennal segment of *Porcellio* and no expression in the maxillary appendages (Abzhanov and Kaufman, 1999a).[17]

[17] *Parhyale* has a small expression domain located in the second antennal segment, however the data are not published in a peer-reviewed journal, are not annotated and the resolution of the photographs

pb has additional conserved expression domains of unknown significance. The *in situ* hybridisations of *mxp* transcripts in chapter four show that *mxp* is expressed in the intercalary/second antennal segment and the mesoderm of the mandibular segment. Mutants for *mxp* do not have observed effects on these segments. *lab* knock down embryo phenotypes that affect the intercalary segment are difficult to interpret (Posnien and Bucher, 2010). I anticipate that loss of both the intercalary and mesodermal domains of *pb* results in phenotypes but the phenotypes will be difficult to detect. Considering that *Dfd, mxp* and *Cx* are able to pattern the gnathal segment appendages in a fractional or additive manner it is possible that *pb* will also be able to pattern mandibular mesodermal derived structures in an additive manner.

The intercalary/second antennal segment expression domain of *pb* is primitive as this expression domain is conserved in non-mandibulate arthropods. In the spider, *pb* homologues are expressed in the pedipalp segment which is homologous to the intercalary/second antennal segment of mandibulates, shown in fig.7.5B (Telford and Thomas, 1998b; Abzhanov et al., 1999; Schwager et al., 2007).

In the onychophoran *Euperipatoides*, an outgroup to the arthropods, the anterior boundary of the *pb* homologue is expressed in the slime papilla segment (fig 7.5A), which is the homologous segment to the intercalary/second antennal segment (Eriksson et al., 2010). In terms of the colinearity of Hox gene expression this makes sense as the *pb* gene is the second Hox gene in the Hox cluster (starting from the 3'end) before *Dfd* and *Scr* and is expressed more anteriorly to these genes (Hughes and Kaufman, 2002a).

Expression of *Scr*

Scr has a more diverse expression pattern than that of other anterior Hox genes, but it is likely that the ancestral anterior limit of expression is in the labial/second maxilla segment (homologous to the third leg segment of chelicerates).

Scr in crickets, centipedes and firebrats is expressed mainly in the labial appendage (and the lacinea endite of the maxilla of insects) (Rogers et al., 1997; Hughes and Kaufman, 2002b). Protein expression of *Scr* is present in early embryonic stages of the mandibular appendage of *Thermobia* (Passalacqua et al., 2010). *Scr* has

is quite poor. Serano, J. M. (http://patelweb.berkeley.edu/JuliaSerano.html). It is clear however that there is no expression in the maxillary segments.

broad expression domains in crustacean species, including the maxillary segments and the maxilliped/first thoracic appendage (Abzhanov and Kaufman, 1999a; Abzhanov and Kaufman, 1999b; Abzhanov and Kaufman, 2000a). In the myriapod *Glomeris, Scr* is expressed in all segments posterior to the mandibular segment (Janssen and Damen, 2006).

Despite this variation of expression, the ancestral anterior limit of expression is likely to be in the fourth post-antennal appendage, homologous to the labial appendage. The anterior boundary of expression of *Scr* in chelicerates is in the third leg segment (Telford and Thomas, 1998b; Abzhanov et al., 1999; Schwager et al., 2007). Therefore the expression of *Scr* in the mandibular and maxillary segments of some mandibulate arthropod species is likely to be derived.

Hox3

Hox3, the homologue of *zen* (which has lost its Hox gene function)*,* is expressed in the mesoderm of the developing mandibular appendage of some mandibulates in a Hox-like manner. In *Daphnia Hox3* is expressed in the developing mandible (Papillon and Telford, 2007) *Hox3* is expressed in the mesoderm of the mandibular and maxillary segments in *Thermobia* (Hughes et al., 2004) and *Glomeris* (Janssen and Damen, 2006). In *Lithobius, Hox3* as well as expression in the mandibular segment, is expressed in the intercalary segment during early embryogenesis (Hughes and Kaufman, 2002b).

The function of *Hox3* has not been studied in any organism however, considering the mesodermal expression of *Hox3* and the conserved ectodermal expression of *cnc* it seems unlikely that *Hox3* will be regulated by *cnc*, and may have an additive role patterning mandibular mesodermal derived structures.

Role of gnathocephalic Hox genes in chelicerates (and onychophorans)

There has been no functional study of anterior Hox gene function in the chelicerates. Expression of the Hox genes in chelicerates is characterized by broad overlapping domains that have their posterior limit at either the posterior of the

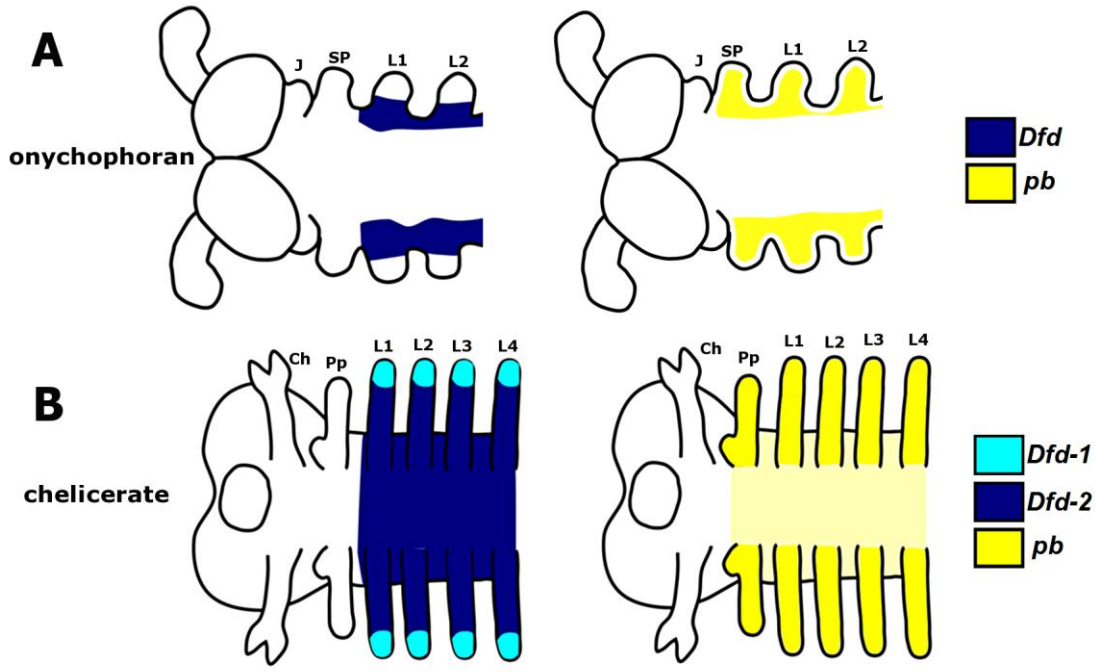

Fig.7.5 Expression of homologs of *Dfd* and *pb* in two outgroups of Mandibulata, the Onychophora and Chelicerata. Onychophoran expression pattern is based on *Euperipatoides* from Eriksson *et al.* (2010). Chelicerate expression is based on *Cupiennius* and *Achaearanea* from Schwager *et al.* (2007) and Abzhanov *et al.* (1999). The anterior expression of *pb* is present in the pedipalp (Pp) segment and the slime papilla (SP) segment which are homologous. The anterior expression of *Dfd* homologues is present in the first leg segment (L1). (A) Expression of *Dfd* and *pb* in an onychophoran *Euperipatoides.* The homologue of *Dfd* is expressed more strongly at the base of the first walking leg and all segments posterior to it. The *pb* homologue is expressed in the mesoderm of slime papilla and all segments posterior to it. (B) Expression of the two *Dfd* homologues, *Dfd-1* and *Dfd-2,* and the homologue of *pb* in chelicerates.*Dfd-1* is expressed in the tips of the walking leg segments. *Dfd-2* is expressed in the remaining part of the leg appendages and the ventral ectoderm.

prosoma or the opisthosoma (see fig. 7.5B). In developing spider embryos, the expression of Hox genes is often specific to different parts of limb which is suggestive of additive patterning functions like those revealed in *Tribolium.* The function of the Hox genes expressed in the prosoma has not been tested to date.

There are two *Dfd* homologues in spiders. *Dfd-1* is expressed in the tips of the L1 to L4 appendages (as shown in chapter six). *Dfd-2* is expressed throughout the L1 to L4 ventral ectoderm and the appendages but is mutually exclusive with *Dfd-1* and excluded from the distal tips of the leg appendages. Consideration of the conservation of proximal appendage expression domains of onychophoran (see fig. 7.5A) and mandibulate *Dfd* Hox gene expression patterns suggests that the spider condition is derived. Interestingly, the L1 to L4 segments of spiders have lost endites on these appendages, and are derived in that respect. One chelicerate group, the xiphosurans (horse shoe crabs) possess endites on their leg segments. Considering that the

mandible is a modified endite it would therefore be interesting to study expression of *Dfd* in representatives of this clade. As I have hypothesized above that *cnc* acquired a function to differentiate the mandibular endite from the maxillary endite, it would be interesting to see if *cnc* is expressed in the endites of the leg appendages of horse shoe crabs.

Originally, it was planned that the homologues of *Dfd* and *cnc* in the spider *Achaearanea* would be knocked down by RNAi to test their function. However, RNAi proved difficult in the spider. As a positive control for the success of RNAi in the spider, numerous attempts were made at knocking down the orthologue of *At Dll*. However, the experiment was not successful and so RNAi of *Dfd* or *cnc* was not pursued further.

The role of cnc in the ancestor to all mandibulates

cnc patterns the mandible by differentiating the mandible from maxillary appendage identity. As the expression of the genes *cnc, Dfd* and *pb* are conserved, there is good reason to suspect that the additive patterning role of these genes in the gnathal appendages of *Tribolium* is conserved in other mandibulates. *Dfd* is expressed in the protopodite of the mandible and maxillae of numerous species. *pb* expression is conserved in the maxillary palps of numerous species. Both *cnc* expression and the repression of *Dfd* expression from the mandibular limb bud are conserved. This strongly suggests that the ancestral role of *Dfd* was to pattern the protopodite, the role of *pb* was to pattern the palp and the role of *cnc* was to differentiate the mandibular protopodite, in particular, the mandibular endite, from maxillary identity.

With consideration of the evidence outlined above, I present a hypothesis of mandible evolution from a molecular perspective. In the ancestor to all mandibulates *Dfd* patterns the base of appendages. *Dfd* expression is conserved in numerous mandibulate arthropods in the mandibular and maxillary protopodites. There is evidence that this condition is primitive for all arthropods, as *Dfd* is expressed at the base of each monopodial limb in an onychophoran (see fig.7.6A).

In the stem lineage mandibulate lacking a characteristic mandibular gnathal edge, *pb* patterns the telopodite and *Dfd* patterns the protopodite of the ancestral second post-antennal limb (the maxilla-like precursor to the mandible). In the ancestor to mandibulate arthropods, the appendages would have likely been serially homologous biramous appendages. There would have been a biramous limb present

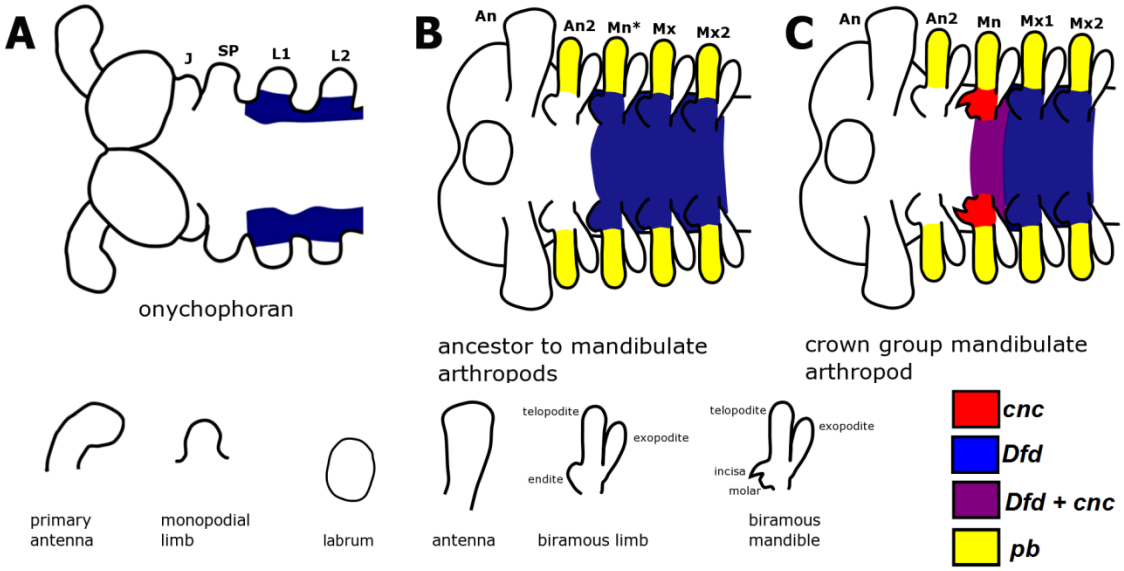

Fig.7.6 Post-antennal expression patterns of *cnc, Dfd* and *pb* in a hypothetical non-mandibulate ancestor to Mandibulata and a hypothetical ancestral mandibulate arthropod. Expression of *Dfd* in an onychophoran (A) may represent the primitive state present in lobopods, the likely ancestral group to all arthropods. (A) Expression of *Dfd* in an onychophoran (B-C) Post-antennal limbs are serially homologous biramous limbs. Whether *cnc, Dfd or pb* is expressed in the exopodite is unknown. (B) hypothetical expression of *cnc, Dfd* and *pb* in a hypothetical non-mandibulate ancestor to Mandibulata (C) expression of *cnc, Dfd* and *pb* in a hypothetical ancestral mandibulate arthropod which is ancestral to all mandibulate arthropods. The mandibular appendage endite is differentiated from maxillary endite identity by *cnc.*

on the second antennal segment. Considering the ancestral anterior boundary of *pb* is likely to be the first post-antennal segment (homologous to the intercalary, second antennal and pedipalp segment) based on expression data from onychophorans and chelicerates, it is not unreasonable to hypothesize that *pb* may pattern the palp of the second antennal appendage and the mandibular palp (compare to the expression of *pb* in a chelicerate shown in fig.7.5B). This hypothetical expression pattern in a non-mandibulate ancestor to Mandibulata is shown in fig. 7.6B. The expression of Hox genes in the exopodite derived palp are unknown.

I hypothesize that *cnc* acquired a new role to pattern the mandibular segment, to differentiate the mandibular endite (and protopodite) from the maxilla to pattern the mandibular gnathal edge in the ancestor to mandibulate arthropods (shown in fig. 7.6C). Based on the results from chapter four, where *cnc* differentiates the mandible from maxillary identity in part probably by the repression of maxillary patterning Hox genes. In this case, the ancestral mandibular appendage is only differentiated from the maxillary appendage by modification of the maxilla-like proximal endite to form a characteristic mandibular gnathal edge comprised of incisor and molar processes.

The primitive mandible most likely possessed both a telopodite and an exopodite. Therefore the ancestral function of *cnc* would have repressed the Hox gene *Dfd* but not *pb* in the mandible, as *pb* in this scenario is required to pattern the mandibular palp.

In the insect lineage, *cnc* has acquired the role of repressing *pb* to repress palp development. It is possible that *cnc* has convergently evolved to repress *pb* in the myriapods which also do not have mandibular palps.

One experiment that could test one aspect of the hypothesis above is to study the expression pattern of the Hox gene *pb* in arthropods that have mandibles with palps. If it was found that mandibles with palps expressed *pb* in the mandibular telopodite it would provide some support for this hypothesis. It is important to state however, that to convincingly demonstrate a likely ancestral state (in terms of gene expression and function), numerous diverse taxa have to be studied, and the different expression patterns plotted onto a phylogeny to evaluate whether each expression pattern represents an ancestral or derived character state.

However, the number of taxa sampled, especially within the crustaceans is quite small. The only studied crustaceans have maxillae that are derived in some respects as well, having missing or greatly reduced telopodites. It therefore remains to be seen whether *cnc* represses the maxilla patterning Hox genes *Dfd* and *pb* in other mandibulates and whether the protopodite patterning function of *Dfd* and the telopodite patterning function of *pb* are conserved.

7.3 General Conclusions

I sought to understand mandible patterning in a model mandibulate arthropod. It was found that *Tc cnc,* like in *Drosophila,* is important for mandible patterning in *Tribolium.* The manner in which *Tc cnc* differentiates the mandible from a maxilla reflects the likely way in which the mandible has evolved in the ancestor to all mandibulates from a maxilla-like precursor that was present in 'crustaceamorph' species like *Martinssonia.* Conserved expression patterns of *cnc, Dfd,* and *pb* suggest that the mandibular and maxillary patterning mechanisms are conserved in mandibulate arthropods.

However, primitive mandibles were in possession of both a telopodite and an exopodite. The mandible therefore evolved from a biramous limb (a biramous maxilla-like precursor) by modification of the proximal endite to form the incisor and molar processes. Both mandibular palps have been lost in the lineage leading to *Tribolium,* as in many other taxa. The maxilla to mandible differentiating role of *Tc cnc* recapitulates the evolutionary history of the appendage but the role of *Tc cnc* to repress palp development in the mandibular appendage is certainly derived.

There has not been a study of the expression of Hox genes in a crustacean with a mandibular palp. The ancestral mandibular appendage undoubtedly had a palp (and was probably a biramous limb). If *cnc* differentiated the mandible from the maxilla in the ancestor to all mandibulate arthropods, the ancestral function of *cnc* would not have been to repress palp development.

As *pb* expression is conserved in the palps of the maxillae of numerous mandibulates, it would be interesting to see the expression pattern of *pb* homologues of crustacean species that have mandibles with palps to see if expression of *pb* is present in the developing mandibular palp telopodite.

As this was the first functional study to examine the mandible patterning genes in a mandibulate arthropod, it is necessary to test function of *cnc* in more mandibulate arthropod taxa, such as the myriapods and crustaceans, to examine whether *cnc* has a conserved role in patterning the mandibular segment.

I also sought to compare mandibular structures to other appendages by studying the expression of genetic markers of appendage segments and the PD domain genes in order to evaluate possible serial homology relationships. Significantly, I discovered genetic evidence of a subcoxal mandibular segment. I also gave molecular evidence for the subcoxal leg segment derivation of the pleuron. Both the subcoxa and coxa of the mandible display significant similarities to the subcoxa of other gnathal appendages, and even the leg appendages. To test that the subcoxal segment is primitive, and that these similarities are evidence of serial homology not convergent evolution, it is important to investigate the expression of the PD domain genes and the Notch signalling pathway in more taxa. Particular appendage types that would be of interest to study would include crustacean mandibles with palps, biramous limbs and the segmented diplopod mandible. By examining the expression of the PD domain genes and Notch signalling, it may be possible to determine if the cardo segment of

the segmented diplopod mandible is homologous to the subcoxa of the *Tribolium* mandible. It may also be possible to determine if the mandibular subcoxa is homologous to the maxillary cardo and the subcoxa of the leg.

The attempt to homologize arthropod appendage segments between taxa and serially homologize appendage segments to other appendage segments is a difficult enterprise. However, it may be possible to determine serial homology between appendage segments by comparison of PD domain gene expression and *notch* signalling pathway expression across numerous arthropod taxa.

It may also be possible to define the protopodite genetically by comparing Notch signalling and PD domain gene expression. The identification of the protopodite in uniramous limbs is more difficult morphologically as the protopodite is defined by the podite to which the telopodite and exopodite are attached, and the exopodite is missing in uniramous limbs. nExd-hth expression was originally used to define the protopodite (Gonzalez-Crespo and Morata, 1996). This was shown to be invalid when it was discovered that *hth* is co-expressed with *Dll* in the trochanter and the discovery that *hth* expressing cells can contribute to the telopodite. Most current analyses exclude the trochanter of the leg from the protopodite. *Tc hth* expression domain extends to the trochanter in the leg, and the first segment of the palp in the maxilla. *hth* is expressed in the first three segments of the uniramous peraeopods (trunk limbs) of *Paryhyale (Prpic and Telford, 2008).* Maybe nExd-hth expression marks the position of the ancestral protopodite.

The expression of PD domain genes together with the expression of the Notch signalling pathway in biramous limbs may be informative in relating the expression of PD domain genes unambiguously to the protopodite. The protopodite is clearly defined morphologically as where the segments to which the exopodite and the telopodite attach.

In order for genetic expression and functional data to contribute towards understanding of the serial homology of limb segments (such as the putative mandible subcoxa), more data from relevant taxonomic groups are required, such as from sister taxa, particularly those with biramous limbs. There has been little attempt to locate the expression of PD domain genes directly to appendage segments in order to homologize different appendage segments across Arthropoda. By studying the co-expression of PD domain genes and the Notch signalling pathway, this may be possible

and may provide new evidence that can resolve long running disputes of segment homology.

Chapter 8:

Materials and Methods

8.1 Animal culture

Tribolium castaneum culture

The strain of *Tribolium castaneum* cultured was the San Bernadino strain (SB), given courtesy of Gregor Bucher (GGNB, Göttingen). Stock cultures of beetles were reared at 25^{o}C (Sanyo incubator, MIR-253) on organic wholemeal flour (Doves farm) supplemented with 5% brewer's yeast (MP Biomedicals, 903312). Egg producing *Tribolium* stocks, pupae producing *Tribolium* stocks and dsRNA injected *Tribolium* beetles were reared at 32 ^{o}C to accelerate development. Flour was replaced every 2-4 weeks.

Achaearanea tepidariorum culture

Spiders were given courtesy of Dr. Angelika Stollewerk (Queen Mary, University of London) and Matthias Pechman (GGNB, Göttingen). Spiders were reared at 25^{o}C in *Drosophila* culture bottles on coconut husk fibre which was maintained at constant moderate level of humidity. Spiders were fed twice a week to once a fortnight. Recently hatched spiderlings were fed wild type *Drosophila melanogaster.* More mature spiderlings were fed *Drosophila pseudoobscura.* Adult spiders were fed *Gryllus assimilis* larval instars. Cocoons were produced by introducing male spiders to mate with female spiders. A cocoon containing about 100-150 embryos would be produced roughly every 5 days.

8.2 Molecular biology techniques.

RNA extraction

RNA was extracted from *Tribolium* or *Achaearanea* embryos for synthesis of cDNA using acid guanidinium-thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987; Chomczynski and Sacchi, 2006) (Ambion, TRI reagent, A9738). Care was taken to avoid contamination of samples with RNases. Surfaces were wiped with RNasezap (AM9780). Quality of the extracted RNA was determined by running samples in denaturing 1% agarose gel in 1x TAE containing guanidium thiocyanate (Goda and Minton, 1995). RNA concentration and purity was determined by analysing samples with a Nanodrop spectrophotometer (Invitrogen, ND-1000).

cDNA synthesis

cDNA synthesis was performed by reverse transcribing RNA using the RETROscript kit (Ambion, AM1710). A minus RT control was performed by using RNA template (without reverse transcription) to check for genomic DNA contamination. Purified RNA that was contaminated with genomic DNA would result amplification of genomic DNA sequences that could include introns.

Primer design

Sequences were obtained by RT-PCR for *Tribolium*. Sequences were obtained by RT-PCR for *Achaearanea* where sequence data was available. In order to obtain longer length sequences from small fragments of available sequence data for *Achaearanea,* RACE was performed.

A 1000bp clone of the *Tribolium* homologue of *Dachshund* (*Tc dac)* was given courtesy of Dr. Nikola-Michael Prpic-Schäper.

Primer sequences

Primer sequences were designed manually using AmpliFX and computationally using primerBLAST and primer3 software. Primers were ordered from Eurofins MWG operon.

Degenerate PCR of *At cnc*

Nested degenerate PCR conditions used for both inner and outer PCRs were as follows: initial hot start 94^{o}C 5 minutes, denaturation step 94 ^{o}C 30 seconds, annealing

step 45^{o}C 45 seconds, elongation step, Final elongation 10 minutes. The PCR cycle was repeated 35 times.

The following products were obtained from combinations of the Inner/nested primers: KVAAQN and KYE/DLTE2 yielded a 71bp product (using SRDEKRA2 and KVAAQN, SRKEKRA2 KRKLDQI1, and KYE/DLTE2 and KRKLDQI1 as outer primer pairs). KVAAQN and PIDEFNE yielded a 110bp product (using SRDEKRA2 and KRKLDQI1 as an outer primer pair).

Polymerase Chain Reaction (PCR)

PCR reactions were performed in an thermocycler (Applied Biosystems, GeneAmp PCR system 2700). PCRs were performed in 50µl reaction volumes. The final concentration of reagents used for the majority of PCRs was as follows: dNTP 0.2mM, Magnesium Chloride 1.5mM, primer concentrations 0.4µM, taq polymerase (Applied biosystems, Amplitaq gold, 2 Units/reaction.

The basic cycling parameters used were as follows: Denaturation step at 94 ^{o}C 5 minutes, followed by a cycle of denaturation at 94 ^{o}C, annealing at various temperatures and an elongation step at 72 ^{o}C. This cycle was repeated 35 times followed by a final elongation cycle of 7 minutes. Annealing temperatures were used initially that were two degrees centigrade below the predicted T_M (calculated using AmpliFX). PCR was optimised by varying annealing temperature if the initial PCR did not succeed.

PCR products were detected by gel electrophoresis by running through a 0.7%-2% agarose gel in 1xTAE with 0.5µg/ml Ethidium Bromide at 4-7V/cm. PCR reactions that produced multiple bands were gel purified by cutting out the band on a UV transilluminator (UVP, model M-20) and extracting the DNA using QIAquick PCR purification kit (Qiagen, 28104) following the manufacturers' instructions. PCR products that produced discreet products were precipitated with 1:10 PCR volume (usually 5µl) 3M Sodium Acetate pH5.2 (Sigma, S-7899) and two times the PCR volume (usually 100µl) of 100% ethanol. DNA was pelleted by centrifuging for one hour at 13,000g. The pellet was washed with 70% (v/v water) ethanol and air dried. The pellet was re-suspended in 20µl distilled water.

Cloning of PCR products

PCR products (within one day of amplification) were ligated into pCRII (Invitrogen, K-2070) or pGEM T-easy (Promega, A1360) plasmid vectors by incubating overnight with T4 DNA ligase at 4 ^{o}C. Ligation reagents were present in the kit provided with the plasmid vector. TOP10 strain of *E.coli* cells (Invitrogen, C-4040) were transformed with ligated plasmids by heat shocking at 42^{o}C for 30 seconds. Transformants were checked by blue/white screening and performing colony PCR of white colonies. PCR products were then analysed by gel electrophoresis. Colony PCR was performed by inoculating a PCR reaction mixture with one white colony. Primers used were M13 forward and reverse and the annealing temperature was 60^{o}C. The PCR products were run on a 0.8-2% agarose gel to check for the presence of an insert of the correct size. Picked transformant clones that were positive for the presence of an insert of the correct size were cultured for one hour in S.O.C. medium and cultured overnight in Lysogeny Broth (LB) at 225rpm at 37^{o}C in a (Thermoscientific MaxQ 4450 barnstead lab line). Bacterial cells were pelleted from LB media by spinning at 2,600g for 15 minutes with a centrifuge (Fisons, Centaur-2 MSE). Plasmid DNA was extracted by using a QIAprep spin Miniprep kit (Qiagen, 27106) and following the manufacturors instructions. Verification of cloned fragments was performed by dideoxy sequencing of products using T7 and SP6 primers. Sequencing was performed out of house by commercial organisations (Source Bioscience). Plasmids were stored at minus 20^{o}C.

Hapten labelled RNA probe synthesis

Labelled RNA probes were transcribed from PCR products from plasmid DNA that contained SP6 and T7 transcription start sites. PCR was performed using T7 and Sp6 primers or M13 forward and reverse primers.

A small aliquot of the PCR was analysed by gel electrophoresis by running through a 0.7%-2% agarose gel in 1xTAE with 0.5µg/ml Ethidium Bromide at 4-7V/cm. The PCR products were purified by precipitating with 5µl 3M sodium acetate and 100µl ethanol at minus 20^{o}C for at least one hour. The precipitated DNA was pelleted by spinning at 13,000g at 4^{o}C for 30 minutes. The pellet was washed with 70% ethanol (in water). The DNA pellet was resuspended with 20µl water. The concentration and purity was determined using a spectrophotometer (Thermoscientific, Nanodrop 2000C).

Three haptens were used to label RNA probes. Digoxygenin (DIG), Fluorescein (FITC) and DNP. Antisense orientation of the cloned gene fragment was determined by DNA sequencing. 300-500ng of the purified DNA was added to a transcription reaction containing transcription buffer, RNase inhibitor. Purified PCR products were transcribed with T7 polymerase (Roche, 10881775001) or SP6 RNA polymerase (Roche, 10810274001) for four hours at 37^{o}C in a water bath. DIG labelled RNA probes were labelled using DIG labelling mix (Roche 11277073910). FITC labelled RNA probes were labelled using Fluorescein labelling mix (Roche, 11685619910). DNP labelled probes were labelled by using a 10x Dinitrophenyl labelling mix (see Solutions and Media below). The transcription reaction mixture was incubated for 4 hours at 37^{o}C. 5µl water was added to the reaction, 1µl was then removed to be analysed on a 0.9% denaturing agarose gel by gel electrophoresis. The labelled RNA probe was hydrolysed by adding 25µl carbonate buffer and incubating at 65^{o}C for 5 minutes. The hydrolysis reaction was stopped by adding 50µl stop solution. The labelled RNA probe was precipitated by adding 10µl 4M lithium chloride, 5µl torula yeast RNA and 300µl 100% ethanol. The labelled RNA probe was pelleted by centrifuging at 13,000g for 30 minutes and then washing the pellet with 70% ethanol. The probe was air dried for 5 minutes and resuspended in 200µl hybridization solution and stored at minus 20^{o}C.

Synthesis of double stranded RNA

dsRNA was transcribed from a PCR template which was flanked by two T7 promoter sequences. These flanker sequences were incorporated by performing PCR with an T7 promoter sequence primer together with an SP6 primer with a T7 promoter attached (T7-SP6 primer). Thereby adding a T7 sequence to one end of the PCR product. The PCR amplification step and purification was the same as for probe synthesis. The PCR was checked by gel electrophoresis and spectrophotometry for correct size, concentration and purity. 500µg-1000µg of PCR product was added to the T7 transcription reaction (Ambion, AM1334, MegaSCRIPT T7 kit). The reaction was performed at 37^{o}C for 4 hours. The dsRNA was purified by lithium chloride precipitation. 30µl 7M lithium chloride and 30µl nuclease free water was added to the transcription reaction and incubated at -20^{o}C for one hour. The dsRNA was pelleted by spinning at 13,000g for one hour. The pellet was washed with 70% ethanol and air dried before being resuspended with 20µl water. A 1µl aliquot was analysed by spectroscopy to check concentration and purity. The dsRNA was denatured and

reannealed by heating at 90^{o}C for 5 minutes then letting the hot plate cool to 70^{o}C (about 15 minutes). The dsRNA was stored at minus 80^{o}C prior to injection.

Degenerate PCR

Most sequences of *cnc* orthologues were retrieved from the Nucleotide database (http://www.ncbi.nlm.nih.gov/nucleotide/).*Ixodes scapularis cnc* orthologue was retrieved from Vectorbase (http://www.vectorbase.org/). Sequences were aligned with the alignment program Maclade and ClustalW. Numerous sets of primers were designed against conserved sequences.

Rapid amplification of cDNA ends (RACE)

Both 5'RACE and 3'RACE were performed using First choice RLM-RACE kit (Ambion, AM1700) according to the manufacturer's instructions. Nested PCR was performed to increase specificity of the PCR reaction.

8.3 *in situ* hybridization protocols.

Tribolium Embryo collection

To collect embryos for fixation, adult beetles were placed on 300µm preseived organic white flour supplemented with 5% brewer's yeast at 32^{o}C. Embryos were sifted from white flour with a 150µm sieve. Germ band extending embryos were obtained 0-24 hours after egg laying on white flour. Germ band retracting embryos were obtained 24-48 hours after egg lay. Very late stage embryos (undergoing dorsal closure) were obtained 48 hours after egg lay.

Fixation of *Tribolium* embryos.

Embryos were dechorionated by immersing in 25% (v/v) bleach and 75% (v/v) distilled water for 5 minutes. Embryos were rinsed in wash buffer and distilled water. Embryos were fixed in fixation buffer with 9.25% formaldehyde for thirty minutes at room temperature at 225rpm on a table top shaker. Embryos were devitellinized by methanol shocking. Fixation buffer was removed prior to adding 100% methanol to the vial. The vial was shaken vigorously for thirty seconds and any embryos that sink to the bottom are collected. Remaining embryos, particularly late stage embryos, were devitellinised by violent aspiration with a 0.9mm syringe. This was repeated several

times. Embryos were stored at -20^{o}C for at least one week before being used for *in situ* hybridization experiments.

Tribolium enzymatic whole mount *in situ* hybridization of *Tribolium* embryos protocol

The whole mount *in situ* hybridization protocol was adapted from a fluorescent *in situ* hybridization protocol (Kosman et al., 2004) and *Tribolium in situ* protocol (Schinko et al., 2009). Embryos were kept in 1.5ml microcentrifuge tubes. All washing steps and incubation steps were performed at room temperature on a rotatory wheel unless otherwise stated. Embryos were given two minutes at the end of every step to settle at the bottom of the eppendorf to minimize loss of germ bands.

Embryos were transferred from methanol to PBS-T and then post-fixed in 4.5% formaldehyde. Proteinase K was added to the embryos at a concentration of 4µg/ml and incubated for 8 minutes. Embryos were rinsed several times over a period of thirty minutes with PBS-T in after the post-fixation and proteinase K digest steps. Post-fixation was repeated at the same concentration. Embryos were washed several times over the period of an hour after the final post-fixation.

Embryos were transferred gradually to hybridization solution. Incubation steps in hybridization solution were performed at 60 ^{o}C in a water bath. The embryos were washed at least four times with pre-heated hybridization solution over a period of at least two hours. The washing steps were performed in a heating block at 60 ^{o}C to ensure constant temperature.

The Digoxygenin (DIG) labelled RNA probe was prepared by adding 0.1µl-5µl to 100µl of hybridization solution. Probe concentration was optimized empirically, but typically optimum probe concentration was 0.5-1µl probe to 100µl Hybridization solution. The probe was denatured by incubating at 80^{o}C for ten minutes and then equilibrated at 60 ^{o}C in the water bath. The embryos were incubated in the probe solution for 42 hours at 60 ^{o}C.

After incubation with the RNA probe, embryos were washed in pre-heated hybridization solution several times over a period of two and a half hours at 60 ^{o}C. Embryos were then washed with PBS-T several times over one hour at room temperature. Prior to antibody incubation, embryos were washed in blocking solution for 30 minutes. Alkaline phosphatase conjugated anti-DIG antibody (Roche, 11 093 274 910) was spun down at 13,000g for 10 minutes at 4 ^{o}C prior to use. Embryos were

incubated with antibody diluted 1 (0.75U/µl):2000 (blocking solution). Antibody incubation was performed on a rocking platform overnight at 4 ^{o}C.

After antibody incubation, embryos were washed at least ten times over a period of 3 hours with PBS-T. Prior to staining embryos with NBT/BCIP, embryos were washed twice with staining buffer. Embryos were stained in the dark in 2% (v/v) NBT/BCIP solution in staining buffer. The staining reaction was stopped by several washes over a period of one hour. Embryos were stored at 4^{o}C overnight prior to secondary staining for double *in situ* hybridizations (see below) or for up to 2 months prior to visualization by microscopy.

Double *in situ* hybridization of *Tribolium* embryos protocol

Double *in situ* hybridization protocol used was the same as the single *in situ* protocol except two probes were added to the hybridization solution, one DIG labelled probe and one Fluorescein (FITC) labelled probe, and the protocol continues after the end of the first staining.

The alkaline phosphatase conjugated anti-DIG antibody was denatured by heating in 1ml of inactivation buffer for 15 minutes at 65^{o}C and left to cool for 20 minutes. Embryos were washed in 50% (v/v) inactivation buffer in PBS-T and then washed several times over a period of 30 minutes with PBS-T. Prior to incubation with alkaline phosphatase conjugated anti-FITC antibody (Roche, 11 428 338 910), embryos were incubated in blocking solution for half an hour. Alkaline phosphatase conjugated anti-FITC antibody was spun down at 13,000g for 10 minutes at 4 ^{o}C prior to use. Embryos were incubated with antibody diluted 1:2000 in blocking solution. Antibody incubation was performed on a rocking platform either overnight at 4 ^{o}C or at room temperature for one hour. After antibody incubation, embryos were washed several times over a period of 3 hours with PBS-T. Prior to staining embryos with NBT/BCIP, embryos were washed twice with FastRed staining buffer. Embryos were stained in the dark in FastRed (Sigma). The staining reaction was stopped by several washes over a period of one hour. Embryos were stored at 4^{o}C for up to 2 months prior to visualization by microscopy.

Tribolium fluorescent whole mount *in situ* hybridization protocol

The fluorescent *in situ* protocol used was very similar to the single *in situ* hybridization protocol with a few modifications. The protocol used was a fluorescent *in situ* hybridization protocol (Kosman et al., 2004). Prior to post-fixation, embryos were treated with xylenes. Embryos were transferred from methanol to ethanol and washed three times in ethanol. Embryos were incubated in xylenes:ethanol (9:1) for one hour at room temperature. Post-fixation steps were increased to 25 minutes. Primary and secondary antibody incubation steps were performed. The washing steps for both antibody steps were the same as for the anti-DIG alkaline phosphatase conjugated antibody step. Numerous hapten, primary and secondary antibody combinations were tried to optimize the signal strength and reduce background (Alexa 405, 488, 555). However, the only data presented in this thesis from fluorescent *in situ* hybridization was using a DIG labelled RNA probe with sheep anti-DIG antibody (Roche (1 333 089) and Alexa 555 Donkey anti-Sheep (Molecular Probes, A-21436).

Tribolium fluorescent whole mount *in situ* hybridization with Tyramide signal amplification.

The tyramide signal amplification (TSA) protocol is very similar to the single *in situ* hybridization protocol with a few modifications. Many of the reagents were provided with the TSA kit #12 HRP-goat anti-rabbit IgG Alexa fluor 488 tyramide (Invitrogen, T20922).

Achaearanea tepidariorum whole mount *in situ* hybridization.

Embryo collection and fixation

Female spiders were mated with males. Cocoons that the female produced were separated. Embryos develop into spiders over the course of one week. Different stages of embryos were obtained by fixing at different times post cocoon production. Embryos were removed from the cocoon and devitenilized by dropping 50% bleach with a pipette for 3 minutes and then washing thoroughly with water. Embryos were fixed in PEMS buffer with 6% formaldehyde for more than 5 hours, often overnight, washed several times with gradually increasing concentrations of methanol (mixed with PEMS). Several washes were performed with 100% methanol and stored at -20^{o}C. Embryos were devitellinized individually using sharpened tungsten wire (by holding over a flame and pulling apart), Dumont no.5 tweezers and Gilson pipettes.

Achaearanea in situ hybridization protocol

The *Achaearanea in situ* hybridization protocol is nearly identical to the *Tribolium in situ* hybridization protocol. Some parameters were altered to try to optimize the *in situ* hybridization experiment: The concentration of probe was tested over a thousand-fold range (0.01µl-10µl labelled RNA probe/100µl hybridization solution). The incubation time of hybridization of the probe to the embryos was varied from overnight to one week. The incubation time of the anti-hapten antibody was varied from overnight to one week. The hybridization temperature was tested over a range of 55^{o}C to 65^{o}C. The concentration of formamide present in the hybridization solution was also increased to 75% (v/v) to reduce the stringency of RNA binding. The number and duration of washes post-hybridization and post-antibody incubation was substantially increased from several washes over two hours to several washes over more than six hours.

Achaearanea Tyramide signal amplification protocol

Achaearanea in situ hybridization protocol is very similar to the *Tribolium in situ* hybridization protocol. Reagents were provided with the Renaissance TSA plus DNP AP system kit (PerkinElmer, NEL746A(AP)).

8.4 Microscopy

Bright field Microscopy.

Prior to taking pictures, embryonic germ bands of *Tribolium* were dissected from yolk using a tool constructed with an eyelash hair attached with candlewax to a Gilson P200 pipette tip. Gene expression revealed by enzymatic *in situ* hybridization of *Tribolium* and *Achaearanea* embryos and cuticle preparations of *Tribolium* larvae was visualized with an Imager M1 microscope (Carl Zeiss). The majority of images were visualized using differential interference contrast (DIC) microscopy using magnification of x50, x100 and x200. Images were taken with Axiocam HRC (Carl Zeiss) and processed using Axiovision product suite Software (release 4.8.2). Images were edited with GIMP 2.6.10. GNU Image Manipulation Program (Copyright© 1995-2008 Spencer Kimball, Peter Mattis and the GIMP Development Team).

Confocal Microscopy

Gene expression revealed by fluorescent *in situ* hybridization was visualized by confocal microscopy using an upright Leica TCS SPE confocal microscope. Laser excitation frequencies used were 405nm, 488nm and 532nm. Images were obtained and edited using Leica application suite (LAS-AF).

Scanning Electron Microscopy

Tribolium embryos were fixed in 9.25% formaldehyde and devitinellized as described for the wholemount *in situ* hybridization protocol. Fixed embryos were rinsed in ethanol and immersed in HMDS and air dried overnight in an embryo dish. Embryos were placed on 0.5" Aluminium pin stubs (Agar scientific). Embryos were sputter coated with gold in an EMSCOPE SC500. Scanning electron micrographs were taken in a JEOL JSM-5410LV scanning microscope at a magnification of 100 to 350 fold. Images were acquired with DigitalMicrograph (Gatan).

Cuticle preparation of *Tribolium* larvae

First instar larvae were collected by placing them on top of 300µm mesh and trapping those that crawl through into 100% glycerol. The larvae were collected and mounted onto a slide with a coverslip with 50% Hoyer's medium and 50% lactic acid and incubated at 70^{o}C for one week. Alternatively, larvae were placed in 90% lactic acid and 10% ethanol and incubated at 70^{o}C for at least one week, and then mounted on a microscope slide to be visualized with DIC microscopy or confocal fluorescent microscopy (larval cuticle auto-fluoresces at all visible wavelengths).

8.5 Parental RNAi in *Tribolium*

Female *Tribolium* pupae were injected with 1-5µg/µl dsRNA. dsRNA was injected with 1mm x 0.78mm borosilicate glass capillaries (CEI Harvard apparatus, GC100TF-10, 30-0038). Needles were pulled by a needle puller (Sutter instrument P-97). Needles were attached to an injection holder (Narishige, HI-7) and attached to a M1 micromanipulator (Helmut Saur). Injected female pupae were added to wholemeal flour plus yeast (5%) with male pupae for 1 week at 32^{o}C. Beetles were then transferred to white flour with 5% brewer's yeast, pre-sieved with a 300µm sieve. Eggs were collected daily for three days and then the beetles were transferred back to wholemeal flour for three days. Embryos were allowed to develop until the 1st instar

larval stage and were collected for cuticle preparations and epistatic interaction assayed by wholemount *in situ* hybridizations with appropriate genes. This cycle of medium transfer was repeated until no observable phenotypes were present.

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Appendix 1:
Solutions and Media

Shown below are the solutions and media that were used in different experiments described above. Small volumes of solutions were filtered with Millex GP 0.22µm filter attached to a 50ml syringe. Larger volumes were filtered with a Nalgene disposable filter unit 0.2µm (450-0020) attached to a vacuum pump (Gast, DAA-V174-ED). pH was measured (where indicated) with a pH meter (Fisherbrand, Hydrus 300). Bacterial media was autoclaved.

Blocking solution:
Western Blocking Reagent solution (Roche, 11921673001) diluted in PBS-T (1:4)

Carbonate buffer (2x):
120mM Na_2CO_3 , 80mM $NaHCO_3$, pH 10.2

Denaturing RNA gel:
TAE buffer with guanidium thiocyanate

Dinitrophenyl (DNP) RNA labeling mix (10x):
DNP-11-UTP (250nmol, 10mM) (Perkin Elmer #NEL555) Ribonucleoside Triphosphate Set (100mM NTPs) (Roche #1 277 057)

Embryo wash buffer:
0.1M sodium chloride, 0.0004% (v/v) Triton X-100

EGTA:
0.5M pH8.0 Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (Sigma E-4378)

Fast Red:
SIGMA FAST Fast Red TR/Naphthol AS-MX Alkaline Phosphatase Substrate Tablets Set red Staining Buffer (Sigma F-4523)

Fast Red staining buffer:
0.1M Tris (water)

Fixation Buffer (*Tribolium):*
1.3x PBS (178mM NaCl, 3.5mM KCl, 1.95mM KH_2PO_4, 8.45mM Na2HPO_4, pH7.4) 67mM EGTA, pH8.0

Fixation Buffer with 9.25% formaldehyde (*Tribolium):*
75% (v/v) *Tribolium* fixation buffer, 25% (v/v) 37% formaldehyde (Sigma, F8775)

Heparin:
10mg Sigma (H-3393) dissolved in 1ml water.

HMDS:
Hexamethyldisilazane (Sigma, 33011)

Hoyer's medium:
54g Gum Arabic (Sigma, G9752), 360g Chloral hydrate (Sigma C-8383), 36g Glycerol (28.6ml) 90ml distilled water.

Hybridization Solution:
50% Formamide (v/v), 5X SSC (750mM Sodium Chloride , 75mM Sodium Citrate), 0.1mg/50ml Salmon sperm DNA, 0.05mg/50ml Heparin, 0.02% Tween-20. Made up to 50ml with water and pH adusted to pH6.5 with concentrated Hydrochloric acid.

Inactivation buffer:
Hybridization buffer with 0.3% (w/v) Sodium Dodecyl Sulphate (SDS)

Lithium Chloride:
4M Lithium Chloride (Ambion, AM9480)

NBT/BCIP:
18.75 mg/ml nitro blue tetrazolium chloride and 9.4 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, toluidine-salt in 67% (DMSO) (v/v) (Roche, 1 681 451 001)

NBT/BCIP Staining Buffer:
Tris 100mM pH9.5, NaCl 100mM, 0.02% (v/v) Tween

Phosphate buffered Saline (PBS):
137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 6.5mM Na_2HPO_4, pH7.4

PBS with Tween:
137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4 , 6.5mM Na_2HPO_4, pH7.4, 0.02% (v/v) Tween

PEMS (pH6.9)
0.1M Pipes, 2mM MgSO4, 1mM EDTA, pH6.9

Proteinase K
10mg dissolved in 1ml nuclease free water (Sigma, P-2308)

Salmon testes DNA:
salmon sperm DNA (Sigma, D-1626) was fragmented by heating at 95^{o}C for one hour.

Sodium acetate:
3M pH5.2 for molecular biology (Sigma, S7899)

SSC (20x):
3M NaCl, 300mM Sodium citrate.

Stop solution:
0.2M sodium acetate, pH 6.0

TAE buffer (x1):
40mM Tris, 20mM acetic acid, and 1mM EDTA

Torula yeast RNA:
10mg/ml Torula yeast RNA (Sigma, R6625)

Appendix 2:

Primer sequences

Shown below are the primer sequences that were used in the different polymerase chain reactions (PCR) and rapid amplification of cDNA ends (RACE) experiments described above.

Table A.1 Sequence of primers used to amplify *Tribolium* partial cDNA.

primer	nucleotide sequence	Gene accession number	PCR product size (bp)
Tc cnc fw	GCAACAGTGGGCCCTATTTA	NM_001170642	2619
Tc cnc rv	GTGGTGGCTCCTTGTGTTCT		
Tc Dfd fw	CCA AGTGAGGAGTACAACCAG	NM_001039421	1142
Tc Dfd rv	TACAAGGCCGTGAGTCCGTAA		
mxp fw	ATAGCTGCTTCGCTAGACCTTA	NM_001114335	1294
mxp rv	TCGCAGGTGGGGTCATTAT		
Tc Dll fw	CAGCAGGTGCTCAATGTGTT	NM_001039439	1051
Tc Dll rv	ATTAAACAGCTGGCCACACC		
Tc hth fw	AGCCGTTTTCTCCAAACAGA	NM_001039400	975
Tc hth rv	GGATAGTGCGCGTACTGGTT		
Tc prd fw	ATGCACAGACATTGCTTTGG	NM_001077622	1171
Tc prd rv	GGATCGTCACAGTGTTGGTG		
Tc ser1 fw	TCCTTCTGCTACTCAACCTGCTAC	XM_964393	1146
Tc ser1 rv	GGGGACATTCGCACTTGAACAT		
Tc ser2 fw	ATTTGGTGCGGTCTGGGAAACT	XM_964393	1058
Tc ser2 rv	TCGGGGTTTTGCGCTTTGTAGA		
Tc ser3 fw	AAGGCAACGTTTGCCAATTCGG	XM_964393	1450
Tc ser3 rv	TCCCATGTGCAACTTCCTGGAGAT		
Tc del fw	CGTGAGCGATAAATGGACCGAAGA	XM_964994	1179
Tc del rv	CCGACCGGACAAAGACACTCAAAA		
Tc N fw	ACTGCGCAATATGGGCAAACAC	NM_001114381	1184
Tc N rv	TCGTTTCCATTTCGGGACTTGTCG		
Tc maf fw	GAAAATCCGAGCGAAAGTCA	XM_001807507	368
Tc maf rv	TGGGATTGGGATTTTGTTGT		

Table A.2 Sequence of primers used to amplify *Achaearanea* partial cDNA.

primer	nucleotide sequence	nucleotide sequence Accession number	product size (bp)
At dfd fw	CAGTATGCTCAAGATCCAAAG	AB433904	1113
At dfd rv	ACTTTAACATGTCGTTCCAGA		
At en fw	ACCTTGGAGTATGGAGTGCG	AB125741	868
At en rv	CAAGGGAATCTGCTGGCATTC		
At dll fw	CGCGAGAGGTAACACGTTCGGA	FM876233	1009
At dll rv	CCGGAGGGCTGACGGTAGCAT		
At cnc fw	ACTCTGTTCCTAAGGCAAAT	-	582
At cnc rv	CATTGGAGTCAGTGTTACCTA		

Table A.3 Sequence of degenerate primers used to amplify the *Achaearanea* homologue of *cnc*.

Primer (amino acid sequence)	Sequence
RDEKRA1	CG CGC GAY GAR AAR CGN GC
RDEKRA2	CG CGC GAY GAR AAR AGR GC
PIDEFNE	CCC ATC GAN GAR TTY AAY GA
KYE/DLTE1	CC AAG TAY GAN CTN ACN GA
KYE/DLTE2	CC AAG TAY GAN TTR ACN GA
KVAAQN	TTC TGN GCN GCN ACY TT
KRKLDQI1	ATY TGR TCY AGY TTN CGY TT
KRKLDQI2	ATY TGR TCY AGY TTY CTY TT

Table A.4 Sequence of primers used to amplify the *Achaearanea* homologue of *cnc* using RACE.

RACE primer	sequence
5' RACE *At cnc 1*	CCTCGCCGACGAATATCTCTTATCAGAGT
5' RACE *At cnc inner 1*	TGAGCCTCTGTCAATTCATATTTTGAAAGTC
3' RACE *At cnc 1*	GACTTTCAAAATATGAATTGACAGAGGCTCA
3' RACE *At cnc inner 1*	ACTCTGATAAGAGATATTCGTCGGCGA
3’ RACE *At cnc 2*	GACTTTCAAAATATGAATTGACAGAGGCTCA
3’ RACE *At cnc inner2*	ACTCTGATAAGAGATATTCGTCGGCGA
5' RACE *At dll*	TGTGCGCGGTTTTCTCATTTTCTTCC
3' RACE *At dll*	ATGTCCATCACCTCCAAGGGACGAT
5' RACE inner *At dll*	ATCGTCCCTTGGAGGTGATGGACA
5' RACE inner *At dll*	GGAAGAAAATGAGAAAACCGCGCACAA
5' RLM-RACE Outer primer	GCTGATGGCGATGAATGAACACTG
3' RLM-RACE Outer primer	GCGAGCACAGAATTAATACGACT
5' RLM-RACE inner primer	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG
3' RLM-RACE inner primer	CGCGGATCCGAATTAATACGACTCACTATAGG

Appendix 3:

Details of *Tc cnc*^{RNAi} experiments

Shown below are the details of *Tc cnc*^{RNAi} experiments performed in chapter four. Table A.5 shows the proportion of *Tc cnc*^{RNAi} knock down phenotypes compared to wild type phenotypes observed during the course of a parental RNAi experiment. Table A.6 shows the correlation between the concentration of injected *Tc cnc* dsRNA and mortality of injected *Tribolium* pupae. Table A.7 shows the numbers of injected *Tribolium* pupae and the concentration of injected *Tc cnc* dsRNA used for *in situ* hybridization experiments.

Table A.5 Numbers of *Tc cnc*^{RNAi} *Tribolium* larvae with knock down phenotype

number of Days post-injection	*Tc cnc* knock down phenotype	wild type phenotype	uncertain
8	29	4	-
9	17	2	-
10	-	2	2
15	-	2	-
19	-	9	-
20	-	14	-
21	-	11	-

Tc cnc dsRNA concentration is unknown for this experiment, but is likely to be 1-2µg/µl based upon observed size of RNA pellet during the dsRNA precipitation step. Female pupae injected: 218. Female beetles eclosed: 195. Survival rate from injection to eclosure: 89.4%. Dead beetles by day 20 : 117 (probably female as females were injected with *Tc cnc* dsRNA which is likely to be responsible for the increased mortality observed). *Tc cnc* knock down phenotype is characterized by the transformation of the mandibles into maxillae. More extreme phenotypes (failure of germ band formation) were present but not counted.

Table A.6. Concentration of injected *Tc cnc* dsRNA and the mortality of *Tribolium* adult beetles

Concentration of *Tc cnc* dsRNA injected	died by day 10 post injection	number of injected female pupae	% mortality[18]
750ng/µl	58	100	58%
500ng/µl	43	100	43%
360ng/µl	55	120	45.8%

Table A.7 Numbers of female *Tribolium* pupae injected with concentrations of *Tc cnc* dsRNA used for collection of embryos for *in situ* hybridization

female pupae injected	*dsRNA* concentration
290	2-3 µg/µl
200	2.8-8.1 µg/µl
200	1.5µg/µl
186	1-2 µg/µl
200	500ng/µl
660	0.5-1.25µg/µl
1736 TOTAL	-

[18] Mortality assuming that dead adult beetles were injected females as females were injected with *Tc cnc* dsRNA which is likely to be responsible for the increased mortality observed.

Appendix 4:
Details of *Tc Dfd*^{RNAi} experiments

Shown below are the details of *Tc Dfd*^{RNAi} experiments performed in chapter five. Table A.8 shows the numbers of injected *Tribolium* pupae and the concentration of injected *Tc Dfd* dsRNA used for *in situ* hybridization experiments. 3-4 µg/µl dsRNA of *Tc Dfd* was injected into 80 females to obtain larvae with knock down phenotypes for cuticle preparation.

Table A.8 Numbers of female *Tribolium* pupae injected with *Tc Dfd* dsRNA

female pupae injected	*dsRNA* concentration
200	1-4 µg/µl
210	1.5 µg/µl
400	1.5 µg/µl
810 TOTAL	**-**

Appendix 5:

Details of additional RNAi experiments

Shown below are the details of parental RNAi experiments in *Tribolium* that were performed. Table A.9 shows the numbers of injected *Tribolium* pupae, the identity of the gene and the concentration of injected dsRNA and observed results of the RNAi experiments.

Table A.9 Additional *Tribolium* parental RNAi experiments

Gene	number of pupae injected	concentration of dsRNA	Mortality and observed effects
Tc dac	481	1.7-4.9µg/µl	Lethal effects observed: 332 dead pupae on slides (69%) mortality, >100 dead beetles in flour, no phenotype in remaining cuticle preparations of larvae.
Tc prd	287	1-1.5 µg/µl	39 dead beetles on slide. Pair rule phenotype observed (deletion of alternate segments), expression of *Tc prd* present in endites, therefore knock down effect wore off during embryogenesis.
Tc kn	80	1-2 µg/µl	No observed phenotype present in offspring of injected female beetles.
Tc maf	60	1-1.5 µg/µl	Embryonic lethal. No embryos developed into larvae.

Appendix 6:
CNC and bZIP family members

Shown below are the CNC and bZIP family member genes from different animal species that were aligned with the sequence obtained by degenerate PCR and RACE in the spider *Achaearanea*, as described in chapter six. Sequences were aligned in order to determine whether the cloned sequence was a true homologue of *cnc*. Sequences were both manually aligned using Bioedit and aligned with the programs Maclade and ClustalW. *cnc* homologues are listed in Table A.10. bZIP family members are listed in Table A.11.

Table A.10 Homologues of *cap'n'collar (cnc)*.

Species	Gene identity	Accession number
Drosophila melanogaster	*cap'n'collar (cnc)*	NM_170053.2
Aedes aegypti	*Aa cnc*	XM_001650266.1
Anopheles gambiae	*predicted gene*	XM_001688113.1
Culex quinquefasciatus	*Cq cnc*	XM_001845372.1
Tribolium castaneum	*Tc cnc*	NM_001170642
Apis mellifera	*predicted Am cnc*	XM_001120971.1
Nasonia vitripennis	*predicted Nv cnc*	XM_001603933.1
Acyrthosiphon pisum	*predicted protein*	XM_001942670
Thermobia domestica	*Td cnc*	AF104004
Daphnia pulex	*cDNA clone CANY2330*	FE327056
Glomeris marginata	*Gm cnc*	AM279684
Ixodes scapularis	putative bZIP family member	XM_002435034
Caenorhabditis elegans	*SKiNhead family member (skn-1)*	NM_171347
Xenopus laevis	*nuclear factor (erythroid-derived 2)-like 1 (NFE2L1)*	NM_001086544
Danio rerio	*nuclear factor (erythroid-derived 2)-like 1 (NFE2L1)*	NM_175043
Homo sapiens	*nuclear factor (erythroid-derived 2)-like 1 (NFE2L1)*	Q14494
Pan troglodytes	*nuclear factor (erythroid-derived 2)-like 1 (NFE2L1)*	XM_001173202.1
Gallus gallus	*nuclear factor (erythroid-derived 2)-like 1 (NFE2L1)*	NM_001030756.1

Table A.11 bZIP family members

Gene	Species		Accession number
AP-1	*Homo sapiens*	bZIP family member	NP_002219.1
Jra (Jun-related antigen)	*Drosophila melanogaster*	bZIP family member	NP_724882
FOS	*Homo sapiens*	bZIP family member	CAG47063.1
kayak (fos-related antigen)	*Drosophila melanogaster*	bZIP family member	NP_001027580
cAMP response element binding protein (CREB)	*Drosophila melanogaster*	bZIP family member	NP_001097018.1

Appendix 7:

Sequence of the homologue of cap'n'collar in *Achaearanea tepidariorum*

Shown below is the cloned sequence of the homologue of *cnc* in *Achaearanea tepidariorum (At cnc*), as described in chapter six, that was obtained by degenerate PCR and RACE and then used to make labelled RNA probes to use in *in situ* hybridization experiments.

At cnc nucleotide sequence obtained from degenerate PCR (72bp):

AGACTTTCAAAATATGAATTGACAGAGGCTCAAATTACTCTGATAAGAGATATTCGTCGGCGAGGAAAAAAT

At cnc nucleotide sequence (1207bp):

CACTCTGTTCCTAAGGCAAATGGATACAACTGTTCAAATTTCTTGCAGCATAATCATACTTACCACATACCCTTCAACAATGATGATCTATGTCACA
AACCAATTACAAGAGACAAGCAAAGATCATATTCTGATGATGATTCTAGCAACAAGGATGAAAAAAGGGCAAAGGAACTCAAACTCCCTATCAG
TATTTCTGATATAATTAATTTAGCCATTGAAGAATACAATGAGAGACTTTCAAAATATGAATTGACAGAGGCTCAAATTACCCTGATAAGAGATAT
TCGTCGGCGAGGAAAAAATAAGGTTGCAGCACAGAACTGTAGAAAACGTAAGATGGATCAAATTAATAACTTGCAACAAGACCTTGATTACCTA
CAAGATGAAAGGATGAAATTGAAATCCAAGCAAAACCATCTGTTGCAGCAAAAGCATGCTATTGAAGAAAAATATGTCCAATTACATGATCTTAT
TTTACAAAATACTAACTGTAGACCACCTAGTGCACCAAGTCTCTCAGCTTTCCATAATTCTCAGAATGCTACATCTGACACTATGTTAGGTAACACT
GACTCCAATGTAAACGGTGGAGCTAAAACAAAAAAGAAATTTAAAAAGTGATAATACATCTATAATTTATTAACCGAATGTGATATACAATTTCTT
ATCGTTAACTACATTTTGATGCCACTCATTTTCATCCATTTATATCATTTGGGAATCATTAAATTTTTTCTTCTTCTAATACATTAATCTGTTAATGCA
TAGATTAAAAAAGTATTTAATGATACTATATCTTTATATTTGTGTTATATTTTATCTTGTACTAAATCACTTTTCTAATAGTACAGTTTTAAGTTGTTA
CTCGCATTTTGTTTTATATTACTTGTATAAATTATGGATCTAAATTGACTTGGCAACAAACAATTTTCATTTAATTTTAATCATTTTACCTCAATCAAT
TTATTTCTTCATAATTATACATAGTTTTTATTTATATTGTTTTCTCTGTTATTGGAATATATTATACAATGGTGTCCTCTGTAATTTTTTTTTCTTTTTA
ATTTTGCATGATTTTTTGATTTGCAAATGATGCCTTATATTTATTTGATTAAACTGTTTTGTTTTCTAATTTGCTATTTTGAAAAGAAAAACATTGTTA
CTTAAACCTTTTTTGATAAAGAAAGAAAATAAAAATATCAGA

At cnc amino acid sequence (207aa):

HSVPKANGYNCSNFLQHNHTYHIPFNNDDLCHKPITRDKQRSYSDDDSSNKDEKRAKELKLPISISDIINLAIEEYNERLSKYELTEAQITLIRDIRRRGKNK
VAAQNCRKRKMDQINNLQQDLDYLQDERMKLKSKQNHLLQQKHAIEEKYVQLHDLILQNTNCRPPSAPSLSAFHNSQNATSDTMLGNTDSNVNGG
AKTKKKFKK